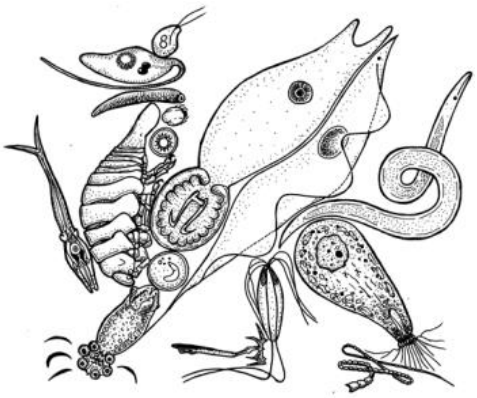
The impact of invasive crayfish on aquatic ecosystems



A thesis submitted for the degree of

Doctor of Philosophy (Ph.D)

by

Joanna James

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DECLARATION AND STATEMENTS

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SUMMARY

Crayfish are keystone species and ecosystem engineers that affect the structure and function of aquatic ecosystems. Whilst ecological impacts are caused by crayfish in their native range, non-native crayfish species typically have a greater effect on some other aquatic organisms and ecosystem processes (Chapter 2). Crayfish are extremely successful invaders that often cause declines in native crayfish (Chapter 3). Of the 7 non-native crayfish species in the UK, the signal crayfish (*Pacifastacus leniusculus*) is currently the most widespread (Chapter 3). Field and laboratory data, however, suggest that in parts of the UK signal crayfish are being outcompeted by more recently introduced virile crayfish (*Orconectes* cf. *virilis*) (Chapter 4). Non-native crayfish also threaten native crayfish through disease, notably crayfish plague (*Aphanomyces astaci*), transmission. Whilst non-native North American crayfish are largely resistant to *A. astaci*, infection in susceptible native European species is usually lethal. Within this study 23 signal crayfish populations were screened for *A. astaci* and 13 were infected (Chapter 5). Virile crayfish from the UK were also infected with *A. astaci*, and therefore should also be considered as a transmission pathway for this pathogen in the UK (Chapter 6). Whilst the majority of studies on crayfish symbionts are focused on *A. astaci*, crayfish host a wide range of micro and macro-parasites. One group of particular interest are branchiobdellidans (Annelida: Clitellata). Two species of these ectosymbionts, *Xironogiton victoriensis* and *Cambarincola* aff. *okadai*, were recently discovered on invasive signal crayfish in the UK (Chapter 7). Owing to their abilities to survive for extended periods off the host and reproduce rapidly both species have a high invasion potential in the UK (Chapter 8). Laboratory experiments show that signal crayfish infested with *X. victoriensis* were less aggressive and poorer foragers than uninfested crayfish, therefore these symbionts may influence signal crayfish invasion dynamics (Chapter 9).

Chapter 1

General Introduction

CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview

More than 640 species of freshwater crayfish have been described, and 5-10 new species are discovered each year (Crandall and Buhay 2008). They are found in all but the Antarctic continent, in a wide range of habitats including rivers, streams, ponds, lakes, wetlands and caves (Crandall and Buhay 2008). Also, some crayfish species spend their entire lives in burrows and are effectively semi-terrestrial (Crandall and Buhay 2008). Crayfish typically occupy a keystone position (Parkyn et al. 1997; Geiger et al. 2005; Crandall and Buhay 2008), interacting with organisms at multiple trophic levels and affecting ecosystem processes through their omnivorous feeding behaviour (Dorn and Wojdak 2004; Geiger et al. 2005; Crandall and Buhay 2008). They also act as ecosystem engineers modifying the structure of the habitat for other organisms by burrowing into the sediment and constructing pits/mounds (Johnson et al. 2011).

Within freshwater ecosystems crayfish are amongst the most widely introduced and successful groups of invasive species (Ilhéu et al. 2007). Globally, invasive species represent the second greatest threat to biodiversity after habitat loss (Wilcove et al. 1998) and have been identified as the leading cause of biodiversity loss in lakes (Sala et al. 2000). Within the USA alone the annual cost of invasive species was estimated at >$137 billion (Pimentel et al. 2000). The introduction of crayfish outside of their native range often has detrimental consequences for the invaded ecosystem. Invasive crayfish often reach larger population sizes and cause greater ecological problems than their native counterparts (Krueger and Waters 1983; Charlebois and Lamberti 1996). This is the case for the signal crayfish (*Pacifastacus leniusculus*), which in its home range (western North America) does not appear to be associated with any environmental problems and has not been observed burrowing into river beds/banks (Shimizu and Goldman 1983). However, in its invaded range (including the UK) signal crayfish burrow extensively (Holdich and Sibley 2009) and have been linked to environmental issues such as localised river bank collapse (Johnson et al. 2011) and the rapid decline of native white clawed crayfish (*Austropotamobius pallipes*) (see Holdich and Sibley 2009; Holdich et al. 2014).

Little is known about the mechanisms that allow crayfish to be more successful in their invasive than their native ranges. The success of a crayfish species when introduced is likely to depend on the life history traits of that crayfish species in comparison to the native crayfish species with which it will compete. For example, signal crayfish introduced into the UK out-compete native white clawed crayfish in individual and population size and growth rate, and have competitively excluded white clawed crayfish from many parts of the UK. Signal crayfish

are, however, now facing competition from a more fecund (Souty-Grosset et al. 2006) crayfish species (the virile crayfish, *Orconectes* cf. *virilis*, see Filipová et al. 2013) recently introduced to the UK.

Overall, this thesis quantitatively reviews the role of crayfish in freshwater ecosystems as keystone species and ecosystem engineers through meta-analysis at a global and national (UK) scale. The importance of crayfish as biological invaders is also evaluated by assessing the comparative ecological impacts of crayfish in their native and non-native range. Given the finding that non-native crayfish have greater effects on freshwater ecosystems, spatio-temporal changes in the density and diversity of non-native crayfishes in the UK are assessed through collating long term distribution records. These data show that invasive crayfish, in particular the signal crayfish (*Pacifastacus leniusculus*) have spread rapidly across the UK, now being ubiquitous throughout England, Wales and Scotland. Considering this, factors potentially influencing the invasion success of signal crayfish in the UK, namely interactions with more recently introduced invasive virile crayfish (*Orconectes* cf. *virilis*) and parasite prevalence/intensity (*Aphanomyces astaci* and *Xironogiton victoriensis*) are also investigated. Combined these data are used to assess the threat posed to British freshwater ecosystems by invasive non-native crayfish species.

1.2 The ecological impacts of crayfish on other aquatic organisms and ecosystem processes

As omnivorous keystone species and ecosystem engineers crayfish interact with organisms on multiple trophic levels (Parkyn et al. 1997; Geiger et al. 2005; Crandall and Buhay 2008; Johnson et al. 2011). At the lower end of the food chain crayfish influence primary producers by reducing the biomass of aquatic plants through consumption and non-consumptive fragmentation (Nyström et al. 2001). In particular, crayfish dramatically reduce aquatic macrophytes sometimes to the point of complete eradication (e.g. Abrahamson 1966; Lodge and Lorman 1987; Elser et al. 1994; Gutiérrez-Yurrita 1998; Nyström et al. 2001; Dorn and Wojdak 2004; Wilson et al. 2004; Rosenthal et al. 2006 Gherardi and Acquistapace 2007). The removal of macrophytes by crayfish may have a detrimental effect on the wider ecological community as these aquatic plants provide food and shelter for many organisms (Lodge and Lorman 1987; Lodge et al. 1994), and contribute to ecosystem functioning through denitrification, oxygenation and nutrient release (Carpenter and Lodge 1986). Ultimately crayfish-induced macrophyte reductions may stimulate the ecosystem to transform from a macrophyte dominated clear water equilibrium to a turbid water balance driven by

phytoplankton (Duarte et al. 1990 cited in Geiger et al. 2005). Therefore the impact of crayfish on primary producers may have ecosystem level consequences.

Crayfish have a pronounced effect on primary consumers, often causing reductions in the abundance and/or taxonomic richness of other macro-invertebrates (e.g. Charlebois and Lamberti 1996; Lodge et al. 1998; Nyström et al. 2001; Stenroth and Nyström 2003; Wilson et al. 2004; Crawford et al. 2006; McCarthy et al. 2006; Flinders and Magoulick 2007; Gherardi and Acquistapace 2007; Bobeldyk and Lamberti 2008; Bjurström 2009; Usio et al. 2009; Bobeldyk and Lamberti 2010; Nilsson et al. 2012). The mechanisms by which crayfish induce reductions in macro-invertebrates include both direct (e.g. predation and resource competition) and indirect effects (through trophic cascades or modification of the physical environment). By employing a selective foraging strategy, crayfish differentially affect macro-invertebrate fauna (Crawford et al. 2006). In particular, crayfish significantly reduce the densities of snails (e.g. Hanson et al. 1990; Lodge et al. 1994; Parkyn et al. 1997; Lodge et al. 1998; Nyström et al. 2001; Dorn and Wojdak 2004; Wilson et al. 2004; McCarthy et al. 2006; Gherardi and Acquistapace 2007; Bobeldyk and Lamberti 2008; Bjurström 2009) and other slow moving invertebrates that are easier for them to capture (Stenroth and Nyström 2003). The invertebrates highly susceptible to crayfish predation are often those associated with slower flow velocities and more tolerant of lower oxygen conditions, therefore crayfish predation may have important implications for river monitoring programmes (Fielding and Constable 2012).

In terms of higher trophic levels, there is strong evidence from wild populations that the abundance of crayfish and some fish species are negatively correlated (e.g. Guan and Wiles 1997; Peay et al. 2009; Bobeldyk and Lamberti 2010). Crayfish can detrimentally affect fish populations through several mechanisms (reviewed by Reynolds 2011) including: predation on eggs (e.g. Savino and Miller 1991; Fitzsimons et al. 2002; Dorn and Wojdak 2004) juveniles (e.g. Rubin and Svensson 1993; Peay et al. 2009; Edmonds et al. 2011) and adults (e.g. Rahel and Stein 1988; Guan and Wiles 1997, 1998; Rogowski and Stockwell 2006) and/or competitive exclusion (Guan and Wiles 1997; Griffiths et al. 2004; Light 2005; Bubb et al. 2009; Peay et al. 2009). Crayfish can evict fish from shelters making them more vulnerable to predation (Rahel and Stein 1988). Exclusion from refuges may also decrease fish growth rates through increased activity levels, energy loss and reduced time available for foraging (Griffiths et al. 2004; Light 2005). Also by forcing fish out into the open water column crayfish may cause an increase in the number of juvenile fish being washed downstream during high flows affecting recruitment of fish for the next generation (Peay et al. 2009). Crayfish presence may, however, benefit populations of predatory fish species that exploit them as a food resource. For instance,

in a small mesotrophic lake in North-East Germany *Orconectes limosus* comprised 48% of annual perch consumption (Haertel-Borer 2005) and invasive red swamp crayfish were the dominant prey item (in terms of occurrence, number and biomass) in Pike diets in the Ruidera Lakes in central Spain (Elvira et al. 1996). Therefore, predicting the ecological impact of crayfish on fish is complicated and likely to be species-dependent.

In wild populations crayfish predation has been linked to amphibian declines, sometimes to the point of localised extirpation (Rodriguez et al. 2005; Cruz et al. 2008). Crayfish prey upon both the eggs and larvae of numerous amphibian species (Gamradt and Kats 1996; Axelsson et al. 1997; Gherardi et al. 2001; Renai and Gherardi 2004; Cruz and Rebelo 2005; Cruz et al. 2006). Predation by crayfish on amphibian eggs does not seem to be prevented by the defensive compounds produced by some amphibian species (Gamradt and Kats 1996; Gherardi et al. 2001). Even those amphibian species that produce eggs with a thick, protective gelatine layer are still vulnerable to crayfish (Gamradt and Kats 1996; Axelsson et al. 1997; Gherardi et al. 2001; Renai and Gherardi 2004), although perhaps less so than those with uncapsulated eggs (Axelsson et al. 1997). Crayfish can also cause sub-lethal damage to tadpoles; for example damage to the tail potentially reducing their swimming ability and making them more vulnerable to future predation attempts (Axelsson et al. 1997).

Through their feeding and locomotory behaviours crayfish affect several ecosystem processes including rates of decomposition and primary productivity. Crayfish, increase decomposition rates by consuming large quantities of detritus (Creed and Reed 2004; Usio and Townsend 2004). The effects of crayfish on primary productivity can be both facultative and detrimental. Crayfish may reduce primary production by consuming aquatic plants and reducing the colonisable substrate available to periphyton (Lodge et al. 1994). Alternatively, crayfish grazing on aquatic macrophytes may increase primary productivity by removing non-autotrophic components of the periphyton matrix and therefore exposing live algal cells to higher light and nutrient concentrations (Charlebois and Lamberti 1996). Crayfish may also elevate primary productivity by excreting large quantities of nutrients, such as ammonia, which may fertilise aquatic plants. Finally by feeding on large quantities of algivorous snails, crayfish may release periphyton from grazing pressure thus indirectly increasing primary productivity (Lodge et al. 1994; Nyström and Abjornsson 2000; Nyström et al. 2001).

Whilst the effects of crayfish on some aquatic organisms (e.g. other aquatic invertebrates and decomposition rates) are clear, their impact on others (e.g. fish density and primary productivity) are more difficult to predict. Also relatively little is known about how the ecological impacts of crayfish vary depending on species, density and endemic status (i.e.

whether or not the crayfish is native to the study region). Whilst further quantification is required, it is generally considered that the impacts of crayfish increase with population density, and are greater for non-native than native species. Indeed these two factors may not be mutually exclusive as non-native crayfish often reach higher population densities than their native counterparts (Krueger and Waters 1983; Charlebois and Lamberti 1996). The comparative ecological impacts of native and non-native crayfish on: a) decomposition rates, b) primary productivity, c) macro-invertebrate density, diversity and biomass, d) fish biomass and refuge use, and e) amphibian egg and larval survival are quantified using a meta-analysis in Chapter 2 of this thesis.

1.3 Crayfish as invasive species

1.3.1 *Global spread and invasiveness*

Freshwater ecosystems contribute 20% (approximately $6.6 trillion, U.S.) to the projected global value of the entire biosphere (Costanza et al. 1997, Gherardi 2010). They also support ~10% of all known species despite occupying less than 1% of the earth's surface (Strayer and Dudgeon 2010). Preserving this biodiversity is challenging in part due to the high susceptibility of freshwater ecosystems to biological invasions which represent the second greatest threat to bio-diversity worldwide (Wilcove et al. 1998).

Globally, some of the most successful aquatic invasive species belong to the subphylum Crustacea (Devin et al. 2005). Within Europe 53% of invasive freshwater species are crustaceans (Karatayev et al. 2009), with crayfish being the most prolific group of invasive aquatic species (Ilhéu et al. 2007; Kouba et al. 2014). Of the 644 described crayfish species 28 have established viable populations outside of their native range (Gherardi 2010). The main invasive crayfish species causing concern are; signal crayfish, spiny cheek crayfish (*Orconectes limosus*), rusty crayfish (*O. rusticus*), red swamp crayfish (*Procambarus clarkii*), Turkish crayfish (*Astacus leptodactylus*) and the common yabby (*Cherax destructor*), (see Table 1.1). There are however, several other species of non-native crayfish that have been introduced to Europe are of an emerging and growing ecological concern (Kouba et al. 2014).

Table 1.1 Most prolific invasive crayfish species worldwide: native and invasive range, adult size (total length, TL, cm) and maximum number of eggs per female per brood (Souty-Grosset et al. 2006; Gherardi 2010).

Species	Native range	Invasive range	Max. adult size	Max. eggs per brood
Astacus leptodactylus	Ponto-caspian basin	Other parts of Europe	15	890
Pacifastacus leniusculus	Northwestern USA and Southwestern Canada	Japan, Europe.	16	500
Procambarus clarkii	Northern Mexico and the South-central USA	Other parts of USA, central and South America, Japan, China, Taiwan, Uganda, Zambia, Europe.	15	600
Orconectes limosus	North America	Other parts of USA, Europe, Morocco	12	600
Orconectes rusticus	Ohio, Kentucky, Tennessee, Indiana and Illinois (USA)	Canada and France	10	575
Cherax destructor	Eastern Australia	Western Australia and Spain	15	450

The introduction of crayfish outside of their native range has taken place in all eco-regions of the world except Antarctica (Austin 1985; Gherardi 2010). Many introductions occurred intentionally for aquacultural (e.g. see Holdich et al. 2014), sometimes as an attempt to alleviate poverty in under developed areas (Gherardi 2006). However, as mobile animals with good dispersal abilities and high fecundity, characteristics associated with successful invaders (MacArthur and Wilson 1967), farmed crayfish inevitably escaped into the wild, often establishing viable populations (Holdich et al. 2014). Unintentionally, the spread of invasive crayfish was facilitated by the use of live crayfish as bait by anglers. In Alberta, virile crayfish appear to have been introduced by angling activities into water bodies up to 400 km from the nearest possible source (Hanson et al. 1990). Thus, these factors combined explain why most invasive crayfish introductions aimed at providing a food source for underprivileged areas have caused more problems than they have solved, referred to as "the Frankenstein effect" (Moyle et al. 1986 cited in Gherardi 2010).

1.3.2 *Interactions with native crayfish*

The introduction of non-native crayfish often results in the displacement of native crayfish species (Lodge et al. 2000; Nakata and Goshima 2006; Olden et al. 2006; Holdich et al. 2014). In extreme cases non-native crayfish introduction may lead to the global extinction of native

crayfish species (see Lodge et al. 2000). The proposed mechanisms of displacement include: competitive exclusion of native by invasive crayfish (e.g. Bubb et al. 2006), disease, notably *Aphanomyces astaci*, transmission (e.g. Unestam and Weiss 1970; Kozubiková et al. 2009; Schrimpf et al. 2013) and reproductive interference (Hill and Lodge 1999; Perry et al. 2001a; Perry et al. 2001b; Perry et al. 2002). The rate at which non-native crayfish displace native crayfish is likely to be elevated if the non-native species is more fecund and reaches sexual maturity earlier than the native species, as is often the case (see Souty-Grosset et al. 2006).

In the UK the introduction of non-native signal crayfish during the 1970s (Holdich and Reeve 1991; Alderman 1996; Peay and Hiley, 2005; Holdich et al. 1999, 2014) corresponded with a rapid decline in native white clawed crayfish (*Austropotamobius pallipes*) populations, sometimes to the point of local extirpation (Sibley et al. 2002; Holdich et al. 2009, 14; Holdich and Sibley 2009; Nightingale 2009). White clawed crayfish populations have decreased so dramatically that this historically abundant (Holdich et al. 2009) native crayfish species has been categorised endangered by the IUCN since 2010 (IUCN 2015). Whilst signal crayfish are currently the most widespread invasive crayfish species in the UK, 6 others have established viable wild populations (see Kouba et al. 2014), exacerbating the threat posed to native white clawed crayfish. The extent to which native crayfish populations have decreased and non-native crayfish populations increased in the UK over recent decades is assessed within Chapter 3 of this thesis.

1.4. Factors influencing non-native crayfish invasion dynamics

1.4.1 *Interspecific interactions between non-native crayfish*

Many European countries now harbour multiple invasive non-native crayfish species (see Souty-Grosset et al. 2006; Kouba et al. 2014). In areas where these invasive non-native crayfish come into contact it is often difficult to predict the outcome of interspecific interactions, and how they may alter the invasion dynamics of one or both species. Elucidating the nature of these interactions is important as ultimately this may determine whether the newly introduced non-native species: a) fails to establish, b) co-exists with the established non-native species or, c) competitively excludes the established non-native species.

As most crayfish species share similar functional niches the strength of competition between established and recently introduced non-native crayfish is predicted to be high (Schoener 1983). Therefore, it is predicted that competitively dominant newly introduced non-native species will displace established non-natives through the process of over-invasion (Russell et al. 2014). Asymmetries in competitive ability may, however, not always be a reliable

predictor of interspecific interactions, as the outcome of these interactions may be complicated by community context (Chase 2003; Duncan and Forsyth 2006; Russell et al. 2014). Established invasive non-native species often have an incumbent advantage, which may prevent a competitively dominant non-native from colonizing, or facilitate their co-existence (Duncan and Forsyth 2006; Russell et al. 2014). It is, however, difficult to assess the extent to which resident invasive non-native crayfish prevent the establishment of novel invaders as unsuccessful species introductions are often not detected.

Of the 7 species of non-native crayfish established in the UK, only 2, the signal (*Pacifastacus leniusculus*, first introduced in the 1970s) and virile crayfish (*Orconectes* cf. *virilis*, introduced around 2004) have overlapping ranges (Ahern et al. 2008; Almeida et al. 2013). Whilst signal crayfish are wide spread throughout the UK (Holdich et al. 2014), virile crayfish currently only persist in 1 watercourse, the River Lee, London (Ahern et al. 2008). Evaluating their interactions with signal crayfish and likelihood of spreading in the UK is critical as they have been confirmed as carriers of *A. astaci* elsewhere in Europe (Tilmans et al. 2014), and are even more fecund than signal crayfish (A. Ellis pers. communication). Spatio-temporal changes in the distribution of signal and virile crayfish species in the River Lee over recent years are assessed in Chapter 4 of this thesis. Within this Chapter competitive interactions between these crayfish species are also investigated.

1.4.2. Host-Parasite Interactions

1.4.2.1 Aphanomyces astaci, *the causative agent of crayfish plague*

Since its first introduction into Europe during the mid-19th century *Aphanomyces astaci*, the oomycete responsible for causing crayfish plague, has spread rapidly across the continent, largely through the movement of non-native North American crayfish species (see Alderman 1996 and Holdich 2003 for reviews). These non-native crayfish are the native hosts of *A. astaci* and usually do not succumb to infection, unless immuno-compromised (Unestam and Weiss 1970; Söderhäll and Cerenius 1999 cited in Kozubíková et al. 2009; Cerenius et al. 2003). Instead they act as vectors and reservoirs of the disease, facilitating its transmission to susceptible European crayfish, in which infection is typically lethal (Unestam and Weiss 1970; Diéguez-Uribeondo et al. 1997; Bohman et al. 2006; Kozubíková et al. 2008, Oidtmann 2012). Therefore, crayfish plague has not only devastated native European crayfish populations but enhanced the survival of non-native North American crayfish by affording them a further competitive advantage.

Whilst crayfish plague is considered as one of the main reasons for the decline of native British white clawed crayfish, the presence of *A. astaci* in the UK has never actually been confirmed using pathogen-specific molecular methods (e.g. Oidtmann et al. 2006; Vrålstad et al. 2009). From a conservation perspective it is important to screen North American crayfish populations for the presence of the pathogen so neighbouring native crayfish populations can be targeted for managed translocation. Efforts to assess the prevalence and infection intensity of *A. astaci* in invasive signal and virile crayfish populations from the UK are detailed in Chapters 5 and 6 of this thesis. Using these data in combination with long term white clawed crayfish distribution records (see Chapter 3) we identified native crayfish populations at high risk of infection with *A. astaci* (Chapter 6). Given that *A. astaci* genotypes differ in virulence (Makkonen et al. 2012), when possible, we also genotyped the strain of *A. astaci* (Chapters 5 and 6).

1.4.2.2 *Ectosymbiotic branchiobdellidans (Annelida: Clitellata)*

Whilst the majority of studies on crayfish symbionts have focussed on *Aphanomyces astaci*, owing to its lethality, crayfish are host to a complex consortium of micro and macro-parasites (see Longshaw 2011 for a review). One group of crayfish symbionts that have gained a particularly notable increase in research interest over recent years are branchiobdellidans (Annelida: Clitellata). These ectosymbionts, common on crayfish throughout the Holarctic (Gelder 1999), are generalists that are directly transmitted between hosts (Govedich et al. 2009; Skelton et al. 2013). Therefore, opportunities for these worms to be co-introduced with their North American crayfish hosts are likely to arise frequently. Indeed branchiobdellidans, non-native to Europe, have been reported infesting invasive crayfish in 7 European countries (Franzén 1962; Gelder 1999; Kirjavainen and Westman 1999; Nesemann and Neubert 1999 cited in Subchev 2008; Quaglio et al. 2001; Oberkofler et al. 2002; Laurent 2007; Subchev 2008; Oscoz et al. 2010; Gelder et al. 2012; Vedia et al. 2014).

Considering branchiobdellidans in the context of crayfish invasions is crucial as their relationship with the host can vary from mutualistic (e.g. Brown et al. 2002, 12; Lee et al. 2009) to commensal (e.g. Keller 1992) to parasitic (e.g. Rosewarne et al. 2012) and therefore they have the potential to influence crayfish invasion dynamics both beneficially and detrimentally. Non-native crayfish populations in the UK have, however, never been screened for the presence of branchiobdellidans. Within this thesis the prevalence and diversity of branchiobdellidans on invasive non-native signal crayfish in Wales is reported for the first time (Chapter 7). Life history traits, including survivorship and reproduction, of these worms are investigated to

evaluate their invasion potential in the UK (Chapter 8). Finally, the relationship between signal crayfish and these co-introduced symbionts is assessed (Chapter 9).

1.5 Thesis aims

- Investigate the comparative effects of native and non-native crayfish on other aquatic organisms and ecosystem processes (Chapter 2).
- Assess spatio-temporal changes in native and non-native crayfish distributions in the UK (Chapter 3).
- Determine how recently introduced non-native virile crayfish (*Orconectes* cf. *virilis*) interact with established invasive non-native signal crayfish (*Pacifastacus leniusculus*) in sympatric regions of the UK (Chapter 4).
- Evaluate the prevalence and infection intensity of the notifiable pathogen, *Aphanomyces astaci*, in signal and virile crayfish populations in the UK (Chapters 5 and 6).
- Develop a risk map for native British crayfish (*Austropotamobius pallipes*) populations, based on the prevalence of *Aphanomyces astaci* in neighbouring invasive non-native crayfish populations (Chapter 6).
- Determine the prevalence and diversity of ectosymbiotic branchiobdellidans (Annelida: Clitellata) on invasive non-native crayfish in the UK (Chapter 7).
- Investigate the potential consequences of branchiobdellidan infestation on the invasion dynamics of non-native crayfishes in the UK (Chapters 8 and 9).

1.6 Thesis layout

This thesis consists of a general introduction on the effects of crayfish on the structure and function of aquatic ecosystems, and their widespread invasive range (Chapter 1). This is followed by a quantitative meta-analysis on the comparative impacts of native and non-native crayfish on numerous aquatic organisms and ecosystem processes (Chapter 2), this has been published in Oecologia. Chapter 3 outlines the compilation of CrayBase, a National database of crayfish distribution records, published in Crustaceana. There are then 6 experimental chapters (Chapters 4-9). These are self-contained chapters all of which are either published (Chapter 4, Marine and Freshwater Behaviour and Physiology; Chapter 7, Aquatic Invasions; Chapter 9, Parasites and Vectors), or in preparation for submission (Chapters 5, 6 and 8). For this reason, there is some repetition of methodological information between chapters. These data chapters are followed by a general discussion, which draws together conclusions from the entire thesis and highlights directions for future work. Finally, the Appendix contains

supplementary material for Chapter 2 and information on side-projects completed during this PhD.

1.7 References

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Chapter 2

Comparing the ecological impacts of native and invasive crayfish: could native species' translocation do more harm than good?

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Comparing the ecological impacts of native and invasive crayfish: could native species' translocation do more harm than good?

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2.1 Abstract

Biological invasions are a principal threat to global biodiversity. Omnivores, such as crayfish, are among the most important groups of invaders. Their introduction often results in biodiversity loss, particularly of their native counterparts. Managed relocations of native crayfish from areas under threat from invasive crayfish into isolated "ark sites" are sometimes suggested as a conservation strategy for native crayfish, however such relocations may have unintended detrimental consequences for the recipient ecosystem. Despite this, there have been few attempts to quantify the relative impacts of native and invasive crayfish on aquatic ecosystems. To address this deficiency we conducted a meta-analysis on the effects of native and invasive crayfish on 9 ecosystem components: decomposition rate, primary productivity, plant biomass, invertebrate density, biomass and diversity, fish biomass and refuge use, and amphibian larval survival. Native and invasive crayfish significantly reduced invertebrate density and biomass, fish biomass and amphibian survival rate and significantly increased decomposition rates. Invasive crayfish also significantly reduced plant biomass and invertebrate diversity and increased primary productivity. These results show that native and invasive crayfish have wide-ranging impacts on aquatic ecosystems that may be exacerbated for invasive species. Subsequent analysis showed that the impacts of invasive crayfish were significantly greater, in comparison to native crayfish, for decomposition and primary productivity but not invertebrate density, biomass and diversity. Overall, our findings re-confirm the ecosystem altering abilities of both native and invasive crayfish, enforcing the need to carefully regulate managed relocations of native species as well as to develop control programs for invasives.

2.2 Introduction

Invasive species are considered to be the second greatest threat to global biodiversity following habitat loss (Didham et al. 2005; Gurevitch and Padilla 2004). Worldwide, invasive species have been identified as a contributing factor in the extinction of 91 species, 34 of which are thought to have become extinct exclusively as a result of biological invasions (Clavero and García-Berthou 2005). The effects of species invasions may be particularly severe in freshwaters, which represent some of the most biodiverse ecosystems (Dudgeon et al. 2006; Strayer and Dudgeon 2010). Within freshwater ecosystems powerful omnivores, such as crayfish, are regarded as among the most ecologically important groups of biological invaders (Strayer 2010). At least 28 species of crayfish are established outside of their native range, and 7 are considered to be invasive (Gherardi 2010). Most notably, American species including signal crayfish (*Pacifastacus leniusculus*) and red swamp crayfish (*Procambarus clarkii*) have been widely introduced across Europe, where impacts upon aquatic flora and fauna have been extensively documented (e.g. Unestam and Weiss 1970; Nyström et al. 2001; Stenroth and Nyström 2003; Bubb et al. 2006; Olden et al. 2006; Bubb et al. 2009; Axelsson et al. 1997).

One of the most notable impacts of invasive crayfish is to drive declines amongst their native counterparts due to competition and/or disease transmission (e.g. Unestam and Weiss 1970; Bubb et al. 2006; Olden et al. 2006). To counter such declines, managed relocations of native crayfish within and outside their natural ranges are often suggested (Olden et al. 2010). Such relocations typically target naturally or artificially isolated water bodies to create 'ark' populations protected from range expansion by invasive species (Haddaway et al. 2012). Relocations have been conducted in parts of the United Kingdom to conserve native white clawed crayfish (*Austropotamobius pallipes*), which have declined rapidly in range and abundance since the introduction of invasive signal crayfish in the 1970s (Sibley et al. 2002; Haddaway et al. 2012). Such relocations of native species outside of their home range raises the controversial issue of whether these new populations should be regarded as invasive and hence viewed as a potential threat to the recipient ecosystem (Olden et al. 2010). Regardless of this, native crayfish display similar polytrophic feeding behaviours to invasive crayfish and so possess the ability to alter the structure and function of ecosystem to which they are introduced. Risk assessments, however, for managed translocations are sometimes ignored in favour of the potential conservation benefits of such actions.

Evaluating the potential detrimental impacts of managed translocation of native crayfish populations is challenging as there is a strong bias in the crayfish literature toward studies on

invasive crayfish species. A recent meta-analysis on the impacts of crayfish on aquatic ecosystems found that invasive crayfish reduced the abundance and/or biomass of aquatic macrophytes and invertebrates and reduced the abundance and/or growth rate of fish and amphibians but did not consistently affect algal biomass (Twardochleb et al. 2013). The impacts of native crayfish were, however only considered in relation to those of non-native species (Twardochleb et al. 2013). Overall, invasive crayfish caused greater reductions in invertebrate and fish biomass and abundance and greater increases in algal biomass than native crayfish, however results were variable and comparisons were only made between specific non-native – native crayfish species pairs (Twardochleb et al. 2013). With the demand for population relocations of native crayfish predicted to increase with intensified pressure from invasive crayfish species it is essential to collate and synthesise all available data on the ecological impacts of native species. Such a meta-analysis will allow the benefits of native species conservation through population translocation to be weighed against the potential risks to wider communities (Olden et al. 2010).

Here, we conduct a global meta-analysis of the published literature concerning the impacts of native and invasive crayfish on a range of taxa and ecosystem processes. We provide a quantitative synthesis of the impacts of crayfish on aquatic ecosystems through testing 3 hypotheses relevant to native crayfish managed relocation planning and invasive species risk assessment: i) native and invasive crayfish perform similar functional roles, i.e. impact the same aquatic taxa and ecosystem process, ii) the magnitude of the impact of crayfish on individual aquatic taxa and/or ecosystem process will be greater for invasive than native crayfish, and iii) the impacts of crayfish on aquatic organisms and/or ecosystems processes will increase with crayfish density.

2.3 Methods

2.3.1 *Study selection and data extraction*

We used keyword searching in Web of Knowledge and Google Scholar to identify peer-reviewed papers quantifying the effects of crayfish on aquatic ecosystems. We also examined reference lists for additional papers, and in some cases contacted authors to gain data that were otherwise unavailable. Literature searches were conducted in 2012. All study types (i.e. laboratory experiments, field mesocosm experiments, field observational studies) and both still and running waters were considered. We examined 132 papers, 44 of which contained relevant studies and reported the following information necessary for inclusion in our meta-analysis: the mean and either standard deviations, standard errors or 95% confidence intervals of the aquatic

organism/ecosystem process in the presence (treatment group) and absence (control group) of crayfish, the sample sizes of each of these groups, and native/invasive status of the crayfish species at the study location (here forth referred to as crayfish status). Of these 44 papers only 35 were used in the meta-analysis (Appendix I) as we excluded aquatic organisms/ecosystem processes for which fewer than 3 independent studies were available. Means and standard deviations/standard errors/confidence intervals were measured directly from figures, through enlarging them and manually calculating values with a ruler if they were not presented within the text or as tables. When reported, information on study duration and density of crayfish was also included, but this information was not mandatory for inclusion in the meta-analysis. The same paper could provide multiple observations for the meta-analysis if independent experiments were conducted using different crayfish species and/or a single experiment measured the effect of crayfish on multiple ecosystem components.

The meta-analysis included 7 taxa or ecosystem processes: i) decomposition rate of dried terrestrial leaf packs (measured as percentage of leaf biomass remaining at the end of the study), ii) primary productivity (measured as chlorophyll *a* production and/or periphyton abundance), iii) the standing crop of macrophytes (referred to as plant biomass), iv) the density or biomass (analysed independently) of benthic macro-invertebrates other than crayfish, v) macro-invertebrate diversity, vi) the biomass and refuge use of fish (measured as the number of fish per shelter), and vii) survival rate of amphibian eggs and/or larvae (measured as the percentage of eggs and/or larvae remaining at the end of the experiment).

To avoid pseudo replication in the principal meta-analysis we applied the following rules (McCarthy et al. 2006): when response variables were measured at multiple time points, only the final observation was used; when experiments included crayfish sex as a factor, data for male crayfish were used (to maximize total sample size as for 61 of the 93 effect sizes calculated from experimental studies only male crayfish were used); when several crayfish densities were studied, we used data from the highest density treatment. However, data for all density treatments were collected and included in a complementary analysis testing whether effect size differed with crayfish density. Additional analyses including crayfish sex as a variable were not conducted, as separate data for female crayfish were only available for 4 out of the 93 effect sizes calculated from experimental studies. For the majority of effect sizes (66 out of 93) calculated from experimental studies the crayfish used were adults. Of the remaining 27 effect sizes 4 were calculated from studies using only juvenile crayfish and the rest from those using crayfish of mixed or indeterminate life stages.

2.3.2 *Effect size calculations*

Following Gurevitch and Hedges (2001), for each observation the effect size (*d*) was calculated as:

$$(1) \quad d = \frac{X_t - X_c}{SD_{pool}} J$$

Where X_t and X_c are the mean values for the treatment and control groups, respectively, and SD_{pool} is the pooled standard deviation, calculated as:

$$(2) \quad SD_{pool} = \sqrt{\frac{(N_t - 1)(SD_t)^2 + (N_c - 1)(SD_c)^2}{N_t + N_c - 2}}$$

Where N_t and N_c are the numbers of replicates, and SD_t and SD_c the standard deviations, for the treatment and control groups, respectively. In equation (1) *J* corrects for small sample sizes and was calculated as:

$$(3) \quad J = 1 - \frac{3}{4(N_t + N_c - 2) - 1}$$

2.3.3 *Statistical analysis*

For each effect size (*d*), we calculated the variance in the effect size estimate, *v* as:

$$(4) \quad \mathbf{v} = \left(\frac{\mathbf{N_t + N_c}}{\mathbf{N_t N_c}} + \frac{\mathbf{d^2}}{\mathbf{2(N_t + N_c)}} \right)$$

From effect sizes (*d*) a weighted mean effect size of the *i* observations was calculated for each ecosystem component as:

$$(5) \quad d^+ = \frac{\sum_i w_i d}{\sum_i w_i}$$

Where each effect size observation is weighted by *w*, the reciprocal of the sampling variance, *v* (see equation 4), and the variance of d^+, v^+ is calculated as:

$$\mathbf{(6)} \quad v^+ = \frac{\mathbf{1}}{\mathbf{\sum_i w_i}}$$

For each ecosystem component we calculated the 95% CI of d^+ as: $d^+ \pm (1.96\sqrt{\mathbf{v^+}})$. This procedure allows the confidence interval to be calculated for weighted mean effect sizes of a single study ($n = 1$; Gurevitch and Hedges 2001). Weighted mean effect sizes (± 95% CI) were calculated separately for native and invasive crayfish and determined as significant if these confidence intervals did not intercept zero.

To test for publication bias across effect sizes normal quantile plots of standardized effect sizes were generated using MetaWin meta-analytical software. Forest plots of individual effect sizes (*d*) were created for each ecosystem component with studies grouped depending on whether the crayfish used in them was native or invasive to the study region (Appendix II).

Linear models were used to investigate factors influencing effect size (*d*). The first model focused upon decomposition rate and primary productivity, whilst the second analysed invertebrate density, biomass and diversity. These categories had larger samples sizes ($n \geq 5$ studies) and were primarily mesocosm studies (c.f. laboratory based studies for amphibians). Grouping the 2 ecosystem processes together, and the 3 invertebrate metrics, maximised the power of the 2 analyses, rather than analysing them individually. The full models regressed effect size upon ecosystem component, crayfish endemic status (native or invasive), crayfish density, study duration and habitat type (whether the study was conducted in a river or a lake). Crayfish species was included as an additional covariate in the decomposition and primary productivity model because of greater sample size of the starting model. Models were fitted using generalized least squares (GLS) because several studies contributed multiple effect size estimates to the analysis: an error correlation structure accounted for potential non-independence of effect sizes from the same studies (Pinheiro and Bates 2000). This meant that we were able to include a larger number of effect sizes (*d*) in the GLS model compared to when calculating weighted mean effect sizes, d^+ (*n* = decomposition [mean effect size: GLS model]: 7:9, primary productivity: 14:19, invertebrate density: 13:15, invertebrate diversity: 8:10, invertebrate biomass: 5:6). For laboratory studies crayfish endemic status was determined on the basis of whether or not the animals were native to the region from which they were harvested. Model fit was assessed using residual plots as recommended by Pinheiro and Bates (2000) and effect size (*d*) was square-root transformed to ensure adequate fit. Models were refined using step wise deletions, manually removing the covariate with the highest *P*-value and re-running the model until only significant terms ($P < 0.05$) remained (Crawley 2007). Models were fitted using R statistical software (version 2.15.2, R development core team 2009).

2.4 Results

The meta-analysis included studies from 4 continents on 12 crayfish species, 8 exclusively in their native range, 1 exclusively in its invasive range and 3 within both their native and invasive ranges (Table 2.1). In general, crayfish densities used in these studies were similar to those observed in wild populations according to the 73 studies where both densities were estimated: study densities for 3 effect sizes were lower than natural population densities, 57 were within

the natural range (albeit often at the higher end of the natural range), and 13 were above the natural range. No evidence of publication bias amongst studies was observed.

Table 2.1 Summary of all papers on native and invasive crayfish used in the current meta-analysis (NB some papers include studies on multiple crayfish species), including information on species of crayfish and the country in which the study was conducted (see Appendix I for full details of the 35 papers included)

Species	Country of study	Number of papers (native + invasive)
Astacus astacus	Sweden	1(1+0)
Austropotamobius italicus	Spain	1(1+0)
Austropotamobius torrentium	Austria	1(1+0)
Cambarus bartoni	USA	1(1+0)
Orconectes marchandi	USA	1(1+0)
Orconectes propinquus	USA	1(1+0)
Orconectes putnami	USA	1(1+0)
Orconectes rusticus	USA	5(2+3)
Orconectes virilis	USA	3(2+1)
Procambarus clarkii	Italy, Spain, Portugal, USA	6(0+6)
Pacifastacus leniusculus	Finland, Japan, Spain, Sweden, UK	12(1+11)
Paranephrops planifrons	New Zealand	1(1+0)
Paranephrops zealandicus	New Zealand	3(3+0)

Analysis of weighted mean effect sizes, d^+ revealed that both native and invasive crayfish significantly reduced invertebrate density and biomass, fish biomass and amphibian survival rate and significantly increased decomposition rates (Fig. 2.1). Invasive crayfish also significantly reduced plant biomass and invertebrate diversity and increased primary productivity (Fig. 2.1). All significant effects on ecosystem components were 'large', based on the conventional interpretation of the magnitude of effect sizes (> 0.8; Cohen 1969). The linear models of individual effect sizes, *d* showed that for decomposition and primary productivity effect size (*d*) was significantly greater for invasive than native species ($F_{1, 25}$ = 6.04, P = 0. 02) and for decomposition rate than primary productivity ($F_{1, 25}$ = 18.80, P = < 0. 001). Effect size (*d*) for these components were not significantly affected by crayfish density ($F_{1, 22}$ = 3.35, P = 0. 08), species ($F_{7, 8}$ = 1.04, P = 0.47), habitat type ($F_{1, 7}$ = 0.08, P = 0.78) or study duration ($F_{1, 15}$ = 0.99, P = 0. 34). For macroinvertebrate studies, there was no evidence that effect size (*d*) significantly differed between density, biomass or diversity ($F_{2, 6}$ = 0.02, P = 0. 98) or was affected by crayfish endemic status ($F_{1, 8}$ = 0.02, P = 0. 91), density ($F_{1, 10}$ = 3.54, P = 0.09), habitat type ($F_{1, 9}$ = 0.21, P = 0.66) or study duration ($F_{1, 13=}$ 1.34, P = 0. 27).

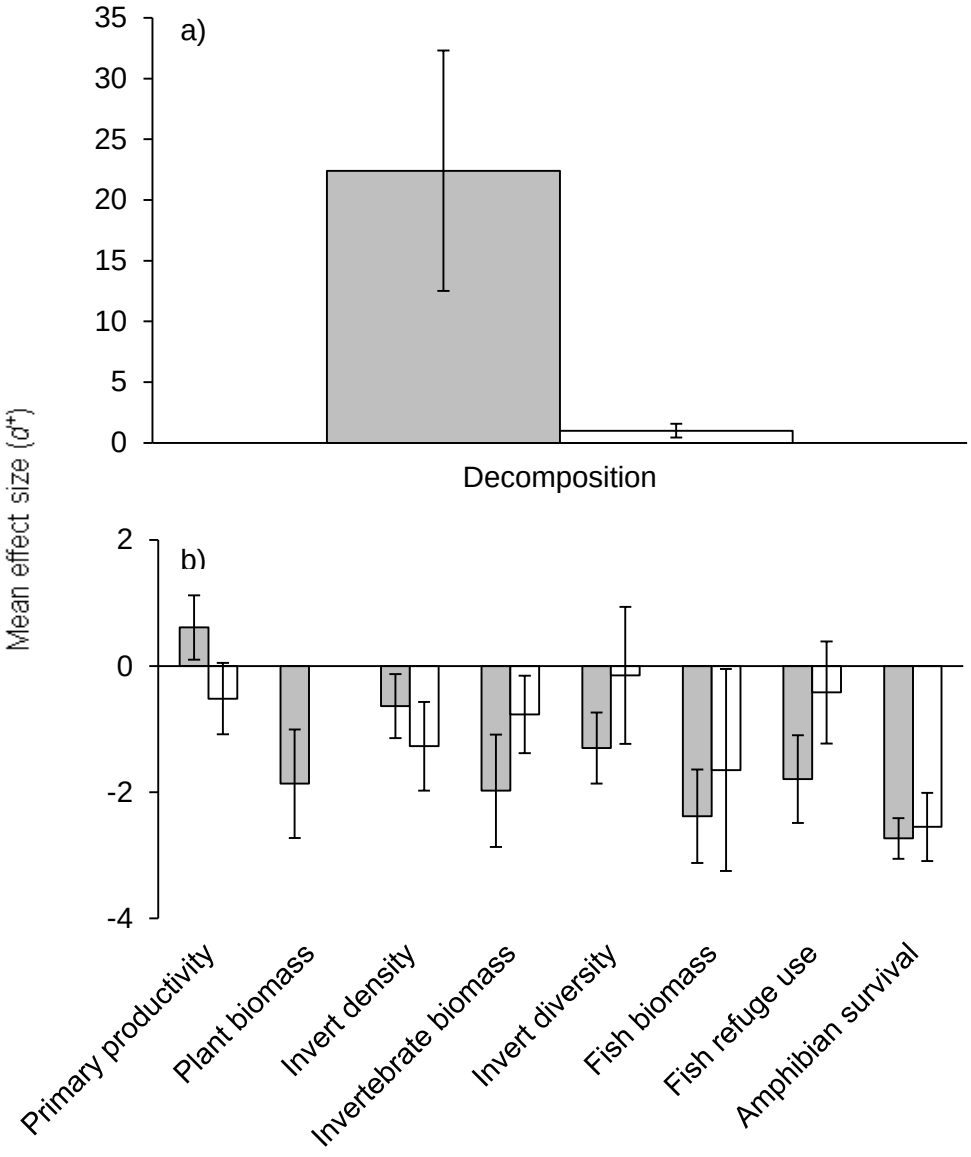

Fig. 2.1 Mean weighted effect size (± 95% CI) of invasive (grey bars) and native (white bars) crayfish on a) decomposition rate and b) plant biomass, primary productivity, invertebrate density, biomass and diversity, fish biomass and refuge use and amphibian egg and larval survival. A negative mean effect size indicates a negative impact of crayfish on that organism/ecosystem process where as the opposite is true for a positive effect size. Where confidence intervals do not intercept zero the result is significant ($P \leq 0.05$)

2.5 Discussion

Our global meta-analysis shows that both native and invasive crayfish have significant and similar effects on ecosystem processes and the abundance/diversity of many aquatic taxa, reaffirming their perceived role as keystone species and ecosystem engineers (Creed 1994; Creed and Reed 2004). This suggests that crayfish, regardless of their endemic status, occupy similar functional roles within freshwater ecosystems.

Both native and invasive crayfish can affect plant and animal communities through predation (e.g. Charlebois and Lamberti 1996; Axelsson et al. 1997; Parkyn et al. 1997;

Nyström et al. 2001; Dorn and Mittlebach 2004; Usio and Townsend 2004; McCarthy et al. 2006; Jackson et al. 2014; Moorehouse et al. 2014), demonstrated by their consistently strong negative effects on plant biomass, other macroinvertebrates, fish and amphibian eggs and larvae. Additionally, crayfish are likely to directly compete with other organisms for resources (Bubb et al. 2009). Indeed the current study provides some evidence of resource competition in finding that the refuge use of fish was significantly lower in the presence of invasive, but not native, crayfish. These direct effects of crayfish on individual ecosystem components may have indirect consequences on other aquatic organisms through trophic cascades (Nyström et al. 2001). For example, by reducing aquatic plant biomass, crayfish reduce the availability of refuges to macro-invertebrates and fish, which may indirectly benefit higher trophic levels (such as predatory fish, birds and otters) by increasing prey vulnerability. Despite evidence suggesting that predation on crayfish increased juvenile otter survival (Ruiz-Olmo et al. 2002) and that invasive red swamp crayfish were readily preyed upon by 4 species of mammalian carnivores and 5 species of ciconiiform birds (Correia 2001), there are currently too few data to properly assess the importance of crayfish as a dietary component for such predators. Our literature review did, however, suggest that crayfish can drive 'top down' trophic cascades. Various studies report that crayfish-induced reductions in invertebrate densities are associated with increases in primary productivity (e.g. Charlebois and Lamberti 1996; Nyström et al. 2001; Bobeldyk and Lamberti 2010). This would be expected as crayfish are known to prey heavily upon algivorous snails (Lodge et al. 1994; Parkyn et al. 1997; Nyström et al. 2001; Bjurström 2009), thereby releasing algae from grazing pressure and facilitating primary production. There are still too few examples to provide a quantitative test of the generality of this phenomenon, highlighting the need for empirical studies to include the effects of crayfish on multiple related ecosystem components.

Invasive crayfish significantly affected a larger number of the ecosystem components investigated than native species, suggesting that they may have greater impacts on freshwater ecosystems. Additionally, the magnitude of the mean effect size (d^+) of invasive crayfish was greater than that of native species on all ecosystem components for which their effects could be directly compared (i.e. everything apart from decomposition and primary productivity) except invertebrate density, which was greater for native crayfish. These results are consistent with the findings of Twardochleb et al. (2013) who also found that invasive crayfish caused greater reductions in the biomass and/or growth rate of other invertebrates and fish, and greater increases in algal biomass than native crayfish. The relative impacts of invasive and native crayfish on decomposition rates, primary productivity, invertebrate diversity and amphibian

egg and larval survival were not evaluated by Twardochleb et al. (2013). Greater impacts of invasive than native species are expected as populations tend to evolve to minimize the negative effects of interspecific interactions on individual fitness (Connell 1980; Futuyma and Slatkin 1983; Rummel and Roughgarden 1985; Shea and Chesson 2002), and community composition should adjust through ecological and evolutionary time to reflect interactions between constituent members (Diamond and Case 1986).

There are at least 4 non-exclusive explanations for invasive crayfish having greater ecological impacts than their native counterparts. First, crayfish species with strong effects may be more likely to be introduced and/or become invasive (Kolar and Lodge 2001; Marchetti et al. 2004; Ricciardi and Cohen 2007). Of the 21 studies on invasive crayfish within the current meta-analysis this only included 4 species. As there are few studies of these crayfish species in their native range, we were unable to assess whether they have consistently strong ecological impacts. Second, invasive crayfish may often be studied in communities that were previously crayfish free, which would confound the comparison of native-invasive effects with community history. Assessing this hypothesis was beyond the scope of the current study. Third, there may be a publication biased towards studies showing a detrimental impact of invasive species. Fourth, invasive crayfish may achieve higher population densities in recipient ecosystems (Parker et al. 2013), possibly owing to release from natural enemies (Torchin et al. 2003). Invasive crayfish are frequently observed at higher densities than natives. For example, invasive rusty crayfish (*Orconectes rusticus*) can reach densities twenty times higher than native species (Krueger and Waters 1983; Charlebois and Lamberti 1996). We found limited evidence that individual effect sizes (*d*) increased with crayfish density ($P = 0.08$ for decomposition/primary productivity model and $P=0.09$ for the invertebrate model), but the sample sizes were modest ($n = 26$ and $n = 23$ respectively). Therefore, from our data, it is unclear how important crayfish density is in determining the impact of crayfish on aquatic ecosystems.

Effect sizes for decomposition and primary productivity varied with crayfish endemic status, with the effect of invasive crayfish being greater than that of natives. This suggests that the impacts of crayfish on these ecosystem processes are variable and highlights that the endemic status of crayfish should be considered when trying to predict this effect. On the other hand the impact of crayfish on other ecosystem components may be more predictable; in the macroinvertebrate model, effect sizes did not differ between invertebrate density, biomass and diversity, nor between native and invasive crayfish. However, investigating the impacts of crayfish on other invertebrates is complicated by the fact that such taxa may be differentially affected by crayfish (Usio and Townsend 2000; Twardochleb et al. 2013). Crayfish

preferentially prey on large soft-bodied invertebrates, such as gastropods (Wilson et al. 2004; Bjurström 2009) but are ineffective predators of highly mobile invertebrate grazers, such as mayflies (Bjurström 2009). Therefore the impacts of crayfish on other invertebrates will vary largely depending on the species composition of the affected community, potentially confounding our comparisons between native and invasive crayfish for these variables. Regardless, our findings imply that translocating native crayfish for conservation purposes could have impacts upon some taxa or ecosystem processes that are of similar magnitude to those of invasive crayfish.

A second important conclusion from this study is the weakness of many aspects of the evidence base for quantifying the effects of invasive and native crayfish. There is a relative paucity of studies quantifying the effects of native and invasive crayfish in a way that can be synthesised with some studies reporting only the results of statistical tests, excluding treatment group means and/or measures of variability that are essential for meta-analysis. For the meta-analysis, only 44 (35 of which were used in the final meta-analysis) out of 132 papers reviewed reported the data necessary for inclusion and these were unevenly distributed across taxa and ecosystem processes (Table 2.1). Within the included studies there is a lack of field experiments and/or observations for certain taxa, in particular amphibians. The value of laboratory studies on interactions between crayfish and these organisms is questionable because in most cases an alternative source of prey is not provided and the ability of the organism to escape crayfish predation is spatially restricted. Concerning the difference between native and invasive crayfish, there are comparatively few studies on the impacts of crayfish in their native ranges, and very few examples of individual crayfish species being investigated in both their native and invasive ranges. These deficits in the empirical evidence emphasise the need for papers to report basic statistics (including control and treatment group means, standard deviations and replicate numbers), and highlights the requirement for further study of native crayfish and ecosystem components which are currently under represented. Studies of individual crayfish species in both their native and invasive ranges are also required to clarify how the impacts of crayfish differ depending upon their native/invasive status. Despite these caveats our results reveal that both native and invasive crayfish have strong ecosystem altering effects, which vary in magnitude across ecosystem components.

2.5.1 *Conclusions and management implications*

Managing and preventing the impacts of invasive species requires the ability to assess the risks associated with current and future biological invasions. Leung et al. (2012) recently proposed

a unified quantitative invasive species assessment framework which considers Transport, Establishment, Abundance, Spread and Impact (TEASI), and meta-analyses like that presented here offer a promising approach for quantifying risk. For most existing and future invasive species, there will not be sufficient species and community specific empirical data to inform case specific decision-making. Meta-analysis can provide broadly applicable quantitative generalisations on the invasion biology of priority taxa.

Once invasive species become established, they are often difficult to eradicate. Combined with the threats of habitat fragmentation and climate change, protecting vulnerable native species may require managed relocations (Olden et al. 2010). Conceptually, such relocations are similar to biological invasions (Shea and Chesson 2002). There is thus legitimate concern that relocating native species may have unintended detrimental consequences on recipient ecosystems (Olden et al. 2010). Our results justify this concern; native crayfish had significant and large effects on numerous ecosystem components that were sometimes larger than those of invasive species. Overall, our findings suggest that the predicted conservation benefits associated with relocating a native species need to be weighed against the potential negative effects on the recipient ecosystem (Olden et al. 2010).

Ultimately, the findings of the current study will contribute to the conservation of freshwater biodiversity as they quantify the ecosystem wide impacts of crayfish and so reinforce the need to develop efficient control mechanisms for invasive crayfish and strictly regulate translocations of native crayfish. By evaluating the general impacts of native and invasive crayfish on freshwater ecosystems this study provides a method of predicting the effects of new crayfish translocations and invasions. Our results help identify priorities for future research, underscore the range of factors that need to be considered when managing native and invasive crayfish, and highlight the potential contribution of meta-analyses in conserving global biodiversity.

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Author contributions

JJ, KAY and JC designed the study. JJ collected the data. JJ, KAY and IPV performed the statistical analyses. JJ wrote the text with comments from all authors.

Chapter 3

A.L.I.E.N. Databases: Addressing the Lack In Establishment of Non-natives databases

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A.L.I.E.N. DATABASES: ADDRESSING THE LACK IN ESTABLISHMENT OF NON-NATIVES DATABASES

BY

J. JAMES[1]), F. SLATER and J. CABLE

3.1 Abstract

Among the principal threats to the conservation of global biodiversity are biological invasions. To monitor their range expansion and develop control programmes, comprehensive, national species' databases need to be created and maintained. This is particularly important for invaders that are known to cause broad and significant ecological problems, such as decapod crustaceans, in particular crayfish. Initiatives such as the UK National Biodiversity Network have recognised the need to promote data exchange and are a valuable resource for collating individual survey records. However, for these data to be used efficiently for research and/or management purposes they need to be combined into national databases. This is challenging and time consuming as individual data-sets are typically in different formats. Here, we compile 25,459 non-native and native crayfish records (reported between 1870 and 2013) from England, Wales and Scotland into 1 database, CrayBase. Such national databases will help facilitate risk assessments for non-native species and promote conservation strategies for indigenous species by identifying populations under the greatest threat from invasives.

3.2 Introduction

The movement of species outside of their native range is a primary concern for biological conservation (Wilcove et al. 1998), which has been implicated in the extinction of at least 91 species (Clavero and García-Berthou 2005). Freshwater ecosystems are particularly vulnerable to the effects of non-native species (Dudgeon et al. 2006). Arguably the most ecologically and economically damaging group of non-native invaders in freshwaters are decapod crustaceans, in particular crayfish (Strayer 2010). Crayfish are omnivorous keystone species (Geiger et al. 2005) and ecosystem engineers (Johnson et al. 2011) that interact with organisms on multiple trophic levels, often with detrimental consequences for the recipient ecosystem. Most notable are the effects of non-native crayfish on their native counterparts that typically decline to the point of localised extirpation (Peay and Hiley 2005; Olden et al. 2006), and in extreme cases global extinction (Lodge et al. 2000).

Each year significant resources are employed in surveying populations of non-native crayfish and/or mitigating associated environmental problems. Remarkably, despite these significant financial and man-power investments, outputs of non-native crayfish surveys are often not maximised with results remaining unpublished and/or inaccessible (Henshall 2012). It is essential that non-native crayfish records are collated in order to monitor temporal changes in their distribution and/or abundance and detect populations of native crayfish under greatest risk.

There have been several previous initiatives focussed on collating the results of species (native as well as non-native) recording schemes. In the UK the first of these was the Biological Records Centre (BRC) that was established in 1964 as a long term partnership between what are now, the Joint Nature Conservation Committee (JNCC) and the Centre for Ecology and Hydrology (CEH). The need to develop a network by which data could be exchanged and made accessible to a wider audience was subsequently proposed in the 'Biological Recording: Need and Network' Report (Berry 1988). This ultimately prompted the creation of the National Biodiversity Network (NBN), which acts a central repository for UK biodiversity data. By 2012 the NBN contained >76 million species records from 160 organisations, including records collated by the BRC.

'Data warehouses', such as the NBN, are invaluable resources for collating and storing biodiversity records. However, greater investment into ensuring that these data are utilised in a way that maximises the effort put into collecting and collating them are urgently needed. In particular there is a demand for records of closely related species to be combined into national databases, in which individual data points have been checked for accuracy and are disseminated

in an accessible and consistent format. Here we create a national database, CrayBase, of non-native and native crayfish species for England, Wales and Scotland and demonstrate how such a resource is invaluable for invasive species risk assessment/management, and native species conservation.

3.3 Methods

Crayfish survey records (presence and absence) were compiled from various sources in England, Wales and Scotland including: Biosys, British Waterways, Dŵr Cymru (Welsh Water), Environment Agency, Fisheries Research Services, Keep Wales Tidy, Natural Resources Wales (formerly Environment Agency Wales, Countryside Council for Wales and Forestry Commission Wales), Powys County Council, Scottish Natural Heritage, Wye and Usk Foundation, and the personal survey records of Drs David Holdich, Peter Sibley and Fred Slater. When available, data were collected on the: date, location (including National Grid References, NGR) and source of the record; species, abundance, (total numbers and Catch-Per-Unit-Effort, CPUE), sex, size, life stage and condition (including signs of disease) of the crayfish caught and, the method and duration of the survey. Any information on other aquatic organisms located during the survey (e.g. if an invertebrate kick sample was performed) and environmental conditions (e.g. flow velocity, channel width, water depth, substrate composition) were included in a comments section of CrayBase. Distribution maps of records in CrayBase were created using ArcGIS 10.0 mapping software.

3.4 Results

To date, 25,459 records of all 8 crayfish species present in England, Wales and Scotland from 1870 to 2013 are included in CrayBase (Table 3.1). Of these 7,543 (7,300 positive and 243 negative) records are of non-native crayfish. In comparison, the National Biodiversity Network database contains 2,708 (all positive) non-native crayfish records across 6 out of the 7 species known from the UK.

Table 3.1. Species of crayfish with wild populations in England and Wales (endemic status, typical size at maturity, origin and suspected data of first introduction for invasive species and date of the first record in CrayBase) (Holdich and Sibley 2009; Adam Ellis pers. communication; Tim Flood pers. communication).

Species name (common name)	Endemic status	Maximum total length (cm)	Origin of introduction	Suspected first introduction	First record in CrayBase
Austropotamobius pallipes (white clawed crayfish)	Native	<12	N/A	N/A	1870
Astacus astacus (noble crayfish)	Non-native	≥15	Germany	1980s	1986
Astacus leptodactylus (Turkish crayfish)	Non-native	≥16	Ponto-Caspian region	1960s	1968
Pacifastacus leniusculus (signal crayfish)	Non-native	≥15	Sweden	1970s	1975
Procambarus acutus (white river crayfish)	Non-native	≥10	Holland	1990s	2013
Procambarus clarkii (red swamp crayfish)	Non-native	≥10	Unknown	1980s	1991
Orconectes limosus (spiny-cheek crayfish)	Non-native	≥12	Unknown	Unknown	2000
Orconectes virilis (virile crayfish)	Non-native	≥12	Unknown	2000s	2012

Native white clawed, *Austropotamobius pallipes* (Lereboullet 1858) and non-native signal, *Pacifastacus leniusculus* (Dana 1852) crayfish are present throughout most of England and Wales whereas the other 6 crayfish species are restricted to geographically isolated populations with limited distributions (Fig. 3.1). In Scotland only signal crayfish are widespread. White clawed crayfish are present in 2 populations in Scotland (Fig. 3.1), where they, ironically, are considered a non-native species (Freeman et al. 2010). Since 2000 the dominant species in the British crayfish fauna has shifted from the native white clawed crayfish to the non-native signal crayfish (Fig. 3.1). The decline in white clawed crayfish populations has been so dramatic that the only populations we can be confident still exist are those for which positive surveys have been reported during the last 5 years (Holdich et al. 2014). There is a lack

of data (both positive and negative) from north and west Wales and the Wash in eastern England, as no surveys have been reported from these regions, despite repeated surveying elsewhere in the UK.

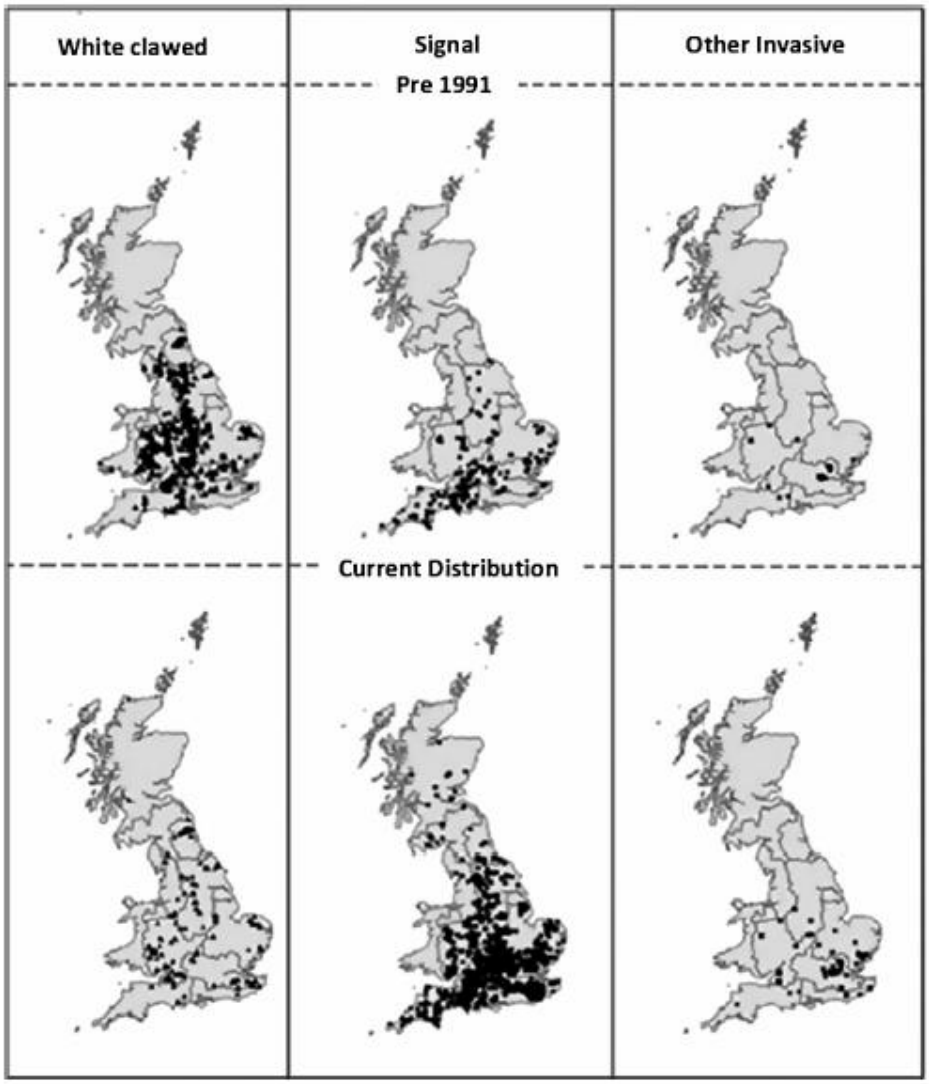

Fig. 3.1. Distributions of native and invasive crayfish in the UK (positive records only). Historical distributions, presented in the upper panel, show records prior to 1991. Current distributions, shown in the lower panel, represent all records in the database with the exception of the native white clawed crayfish which only shows those from 2009-2013, as these are the only populations that we can be confident still exist. Other invasive species include Turkish, noble, spiny-cheek, virile, red swamp and white river crayfish. For all maps each marker represents a single record (although many individual records have the same grid reference and are overlaid).

3.5 Discussion

The creation of CrayBase highlighted the following challenges associated with collating meta-data: a) individual data-sets are presented in different formats some of which require contacting the author to interpret; b) information within some data-sets is not presented in an

analysable format, specifically facts being grouped into a single 'comments' column; c) positive and negative survey records are often combined, which may not be immediately clear; d) individual records need to be checked not only to remove erroneous data but also as vital information, such as whether the survey results are positive or negative, is often only included in the 'comments' section of the data-set. These factors combined make the collation of separate data-sets into an individual database extremely time consuming, and therefore not always feasible.

The large number of invasive crayfish records in CrayBase, almost three fold that in the National Biodiversity Network (NBN), reinforces the fact that many individuals/organisations do not upload their records into data sharing networks. Some records may not have been widely disseminated, as ecological consultants could have confidentiality clauses in their contracts. Therefore, there is a need for the development of legislation and/or other regulatory requirements for all species records to be centrally banked by data repositories, such as the NBN. The records entered into CrayBase were gathered through sending relevant parties direct data requests which stated that all records sent would be incorporated into a national database. To avoid issues with data ownership information on the original source of individual records, when provided, is included in CrayBase. Adopting this direct approach for data collection is time consuming, however it could be employed for target/keystone species, with data then fed back into biodiversity networks, such as the NBN. An individual/organisation with suitable expertise could be responsible for creating the national database for a particular group of non-native species, involving processing data requests and serving as a single point of reference for submitting new/updated records. This is being performed to an extent by Local Biological Records Centres but only at a regional level.

National databases, like CrayBase, will provide a valuable resource for governmental organisations, environmental consultants and researchers to monitor the range expansion of non-native species, and evaluate the risk of furthering their spread when conducting river engineering, conducting surveys or carrying out field experiments. They will also provide a basis for identifying populations of indigenous organisms at greatest threat from non-native species (through geographical distance or water-body connectivity) and hence assist in the targeting of conservation programs. For instance, it is a widely held view that the only way of ensuring the sustainability of native crayfish in the UK is through the translocation of populations to isolated 'ark sites' free from non-native crayfish (Whitehouse et al. 2009). CrayBase could be used to assess the risk of proposed 'ark site' locations from neighbouring non-native crayfish populations.

We encourage individuals and organisations working on British crayfish to contribute to the future development of CrayBase (which will be added to the NBN) by sending new or updated crayfish survey records to the corresponding author of this study over the next 5 years, after which records should be sent directly to the NBN. These records should be submitted in a format consistent with CrayBase and to facilitate this a template spreadsheet is available from the corresponding author. We also suggest that inclusion criteria for crayfish survey records should be standardized and that the minimum information reported should be the: study date and location (including NGR); crayfish species and abundance (total number caught and CPUE where appropriate); survey method and effort (length of manual survey or number of trapping days). Already CrayBase has shown that there is a complete absence of UK crayfish surveys in some areas (west Wales, parts of East Anglia and the East Midlands) and we urge individuals responsible for commissioning biodiversity monitoring surveys to address these discrepancies. Overall, this study shows that the creation of national scale databases is essential to maximise future financial investments into surveying native and non-native species.

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Author contributions

JJ and JC designed the study. JJ created the database and distribution maps. JJ and JC wrote the text. All authors commented on the text.

Chapter 4

Over-invasion in a freshwater ecosystem: newly introduced non-native virile crayfish outcompete established invasive signal crayfish

CHAPTER 4: Over-invasion in a freshwater ecosystem: newly introduced non-native virile crayfish outcompete established invasive signal crayfish

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Over-invasion in a freshwater ecosystem: newly introduced virile crayfish (*Orconectes virilis*) outcompete established invasive signal crayfish (*Pacifastacus leniusculus*)

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4.1 Abstract

Biological invasions are a key threat to freshwater biodiversity, and identifying determinants of invasion success is a global conservation priority. The establishment of introduced species is predicted to be hindered by pre-existing, functionally similar invasive species. Over a 5-year period we however, find that in the River Lee (UK), recently introduced non-native virile crayfish increased in range and abundance, despite the presence of established alien signal crayfish. In regions of sympatry virile crayfish had a detrimental effect on signal crayfish abundance but not vice versa. Competition experiments revealed that virile crayfish were more aggressive than signal crayfish and outcompeted them for shelter. Together these results provide early evidence for the potential over invasion of signal crayfish by competitively dominant virile crayfish. Based on our results and the limited distribution of virile crayfish in Europe, we recommend that efforts to contain them within the Lee catchment be implemented immediately.

4.2 Introduction

Biological invasions are a principal threat to global biodiversity (Wilcove et al. 1998). Despite heightened awareness of the ecological and economical costs of alien species, and legislation restricting species' movements (e.g. Wildlife and Countryside Act 1981), invasion rates continue to increase (Ricciardi 2001; Blackburn et al. 2011). While there is now a large

literature on alien-native species interactions, interspecific interactions between invaders can alter the invasion dynamics of 1 or both species and subsequently affect their impacts on resident biota (Russell et al. 2014). Given that early intervention is critical for managing the impacts of invasive species (EU regulation 1143/2014), identifying the nature of interactions between established and new alien species is a global research priority.

It is difficult to predict the nature of alien species interactions and how they may alter invasion dynamics and the cumulative impacts of serial invasions. Speciose communities are predicted to be more resistant to invasion through increased competition and reduced niche availability (Elton 1958; Shea and Chesson 2002). While there is some theoretical and empirical support for this Biotic Resistance Hypothesis (Elton 1958; Moulton and Pimm 1983; Case 1990), other studies do not support this hypothesis (e.g. Jeschke and Strayer 2005; Pino et al. 2005; Leprieur et al. 2008), and many speciose ecosystems have been, and continue to be, invaded by multiple alien species (Kaufman 1992; Hall and Mills 2000; Ricciardi 2001). The Invasional Meltdown Hypothesis posits that positive interactions (i.e. mutualistic and commensal) interactions among invaders initiate positive population-level feedback that intensifies impacts and promotes secondary invasions (Simberloff and Von Holle 1999; Shea and Chesson 2002). Such facilitative interspecific interactions may be as common as detrimental interactions (i.e. competition/amensalism) (Simberloff and Von Holle 1999; Ricciardi 2001).

Alien species' interactions will depend in part on their functional niches (Russell et al. 2014), because niche overlap should correlate to the strength of exploitative and/or interference competition (Schoener 1983). Competitively dominant species are predicted to have higher population growth rates, and potentially displace subordinate species through the process of over-invasion (Russell et al. 2014). The outcome of such interactions, however, may be complicated by community context (Chase 2003; Duncan and Forsyth 2006; Russell et al. 2014). Established alien species may have an incumbent advantage, which may prevent a competitively dominant invader from colonizing, or facilitate their co-existence (Duncan and Forsyth 2006; Russell et al. 2014).

Among the most widely introduced, ecologically damaging and economically costly groups of alien species are freshwater crayfish (Strayer 2010; Lodge et al. 2012; Twardochleb et al. 2013; Chapter 2). Multiple species of ecologically similar alien crayfish are often introduced into the same region, and interspecific interactions likely influence invasion dynamics. Of the 7 species of alien crayfish established in the UK, only 2, signal (*Pacifastacus leniusculus*, first introduced in the 1970s) and virile crayfish (*O.* cf. *virilis*; see Filipová et al.

2010, introduced around 2004) have overlapping ranges (Ahern et al. 2008; Almeida et al. 2013; Chapter 3). These species exhibit dietary niche partitioning (Jackson et al. 2014), which may facilitate co-existence, at least on a short term basis. Competitive interactions between these species are likely to affect their invasion dynamics and collective impact on recipient ecosystems.

Here, we report the results of a long-term field study and complementary laboratory experiments to link interference competition to the population dynamics of signal and virile crayfish in an incipient zone of sympatry. Specifically, we: 1) quantified temporal changes in the distributions and densities of the species in their sympatric range, 2) tested for asymmetries during dyadic competitive interactions, and 3) determined if asymmetric interference competition predicted the acquisition of refuge habitats.

4.3 Methods

4.3.1 *Field surveys*

Signal and virile crayfish populations were surveyed during the summer (June-September) at fixed sites along a ca. 60 km reach of the River Lee, London, UK in 2006 (19 sites) and 2011 (18 sites) using standardised trapping protocols ('trappy traps' baited with dried cat food and/or trout pellets and checked daily for 2 days) (Fig. 4.1). To estimate spatiotemporal patterns of crayfish abundance in the study reach, catch-per-unit-effort (CPUE) was calculated for each species as: (the total number of crayfish caught/number of traps)/number of trap nights. Using trap data to estimate abundance could be biased in areas of sympatry if 1 species is deterred from entering traps by the presence of the other, however both species were regularly captured in the same trap during the current study, and have previously been used successfully in a range of crayfish surveys (e.g. Wilson et al. 2004; Schrimpf et al. 2013; Sargent et al. 2014).

4.3.2 *Experimental animal collection and maintenance*

In 2013, signal and virile crayfish were collected from allopatric sites in the River Lee. Animals were transported to Cardiff University and housed in separate species tanks (100 L with a crayfish density of ca. 15 individuals/m^2) filled with dechlorinated water (14 ± 1°C) under a 16 h: 8 h light/dark regime. All experiments were conducted under these environmental conditions. Housing tanks had gravel substrate (2 cm depth) and sufficient refuges (plastic tubes and plant pots) for all animals. Crayfish were fed daily with Tetra Crusta crayfish food pellets and 50% water changes were performed weekly. Crayfish with regenerating or missing chela were not used in any experiments, except for the shelter preference trials, for which chelae number was

included as a factor in the analysis. Following experiments, animals were humanely killed by freezing at -20°C, in accordance with the Wildlife and Countryside Act 1981.

4.3.3 *Shelter preference of individual crayfish*

To determine whether signal and virile crayfish utilise shelter differently in the absence of competition, individuals (n = 30 animals per species, of which approx. $^{2}/_{3rds}$ were males, mean [range] carapace length, CL (mm): of 44.7 [36-61] and 46.3 [35-55] for signal and virile crayfish respectively) were placed in a 100 L aquarium with 4 different refuge types (offering different levels of shade) randomly placed in each of the 4 corners: a) hollow PVC tubes sealed at one end with black plastic to simulate dark burrows (L15.5 cm x Dia. 6 cm), b) plastic mesh tunnels simulating partially shaded burrows (L21.0 cm x Dia. 7 cm), c) strips (L30 cm x W1 cm) of black plastic anchored with a weight mimicking aquatic plants, and, d) half of a plant pot cut lengthways (Dia.: 10.5 cm at the base and 16 cm at the entrance) and positioned horizontally to allow crayfish to shelter underneath.

At the start of each trial, a single crayfish was placed at the centre of an aquarium within a small circular plastic holding container. Following a 3 min acclimatisation period, the holding container was lifted and the crayfish was allowed to explore the experimental arena for 16 h. All trials started at 18:00 h (4 h prior to the onset of the dark phase) and terminated at 10:00 h the next day (4 h following the onset of the light phase), at which point the position of the crayfish in the experimental arena was recorded. Trials were run overnight to allow crayfish (which are primarily nocturnal) to explore each refuge type and then take residence within the preferred refuge type following the onset of the light phase. Between trials, the experimental aquaria and all refuges were wiped with 70% ethanol and rinsed thoroughly with water to remove chemical cues.

4.3.4 *Competition and shelter use during dyadic interactions*

The effects of interspecific interference competition on crayfish behaviour and shelter use were assessed in a glass aquarium (L60 cm x W30 cm x D30 cm) containing a mobile plastic divider. To prevent crayfish behaviour being influenced by external stimuli the outer walls and base of the experimental tanks were covered. In each trial (n = 27), 2 crayfish were sex and size matched to within 10% average chelae length (mm). Crayfish were individually marked using coloured nail polish on the dorsal carapace to facilitate species recognition in video footage. Prior to trials commencing, the crayfish were separated by the plastic divider and after a 3 min acclimatisation period the divider was lifted and the crayfish allowed to interact. Trials were

conducted during the light phase when competition for shelter is highest. Crayfish interactions were recorded using Micropix USB cameras and the numbers of 2 aggressive (threat display i.e. chelae raising but no physical contact, and fight initiation i.e. chelae locking) and 2 passive (avoiding i.e. clearly moving away from an approaching threatening animal, and retreating i.e. evading from a physical encounter) behaviours were tallied over a 30 min period. These behaviours were selected following Bergman and Moore (2003) and Alonso and Martínez (2006). Following the 30 min of recording agnostic interactions, a shelter (plastic tube as above) was placed at the end of the tank opposite to where the crayfish were introduced, and equidistant to both animals. Although in allopatric shelter use experiments crayfish of both species preferentially used plastic plant pots, these could not be used for competition experiments as they allowed more than 1 crayfish to shelter beneath them. Following the introduction of the shelter the positions of the crayfish were recorded hourly for 10 h. From these data we calculated the percentage of the 10 observations each crayfish spent sheltering.

4.3.5 *Statistical analysis*

All analyses were conducted using R statistical software (version 2.15.2, R Development Core Team 2009). Generalised Linear Models (GLMs) were assessed using residual plots as recommended by Pinheiro and Bates (2000) and Thomas et al. (2013).

Wilcoxon matched-pairs tests were used to determine if the CPUE of signal and virile crayfish along the River Lee changed significantly between 2006 and 2011. Only those sites where animals were trapped at the exact same locations in 2006 and 2011 were included in these analyses (n = 16). To test whether the presence of 1 crayfish species affected the abundance of the other we calculated the difference in CPUE of each species between 2006 and 2011 in allopatric and sympatric sites, and compared these using Mann-Whitney tests.

For the shelter preference experiment, a GLM with a binomial family and clog-log link function was used to test whether the shelter use (i.e. whether the crayfish were in or out of shelter at the termination of the trial) of crayfish (across all shelter types) depended on species, sex, carapace length or chelae number. For this model, non-significant terms were sequentially deleted from the starting model using Analysis of Variance (Crawley 2007), and only significant terms are reported. A Fisher's test was used to determine if signal and virile crayfish utilised the 4 presented refuge sources differently. Additionally, a Chi-square test was used to confirm that the shelter type used in the subsequent competition experiment, the plastic tube, was selected at similar frequencies by signal and virile crayfish.

Negative binomial GLMs (log link function) were used to investigate whether, for each behaviour type, the number of behaviours performed by crayfish was dependent upon species, sex and size (chelae length, mm). The total number of all behaviours performed by each crayfish was also including as a controlling variable in all models. Separate GLMs were run for each behaviour measured.

To test for differences in shelter acquisition by signal and virile crayfish during dyadic competition trials, we calculated the percentage of the 10 observation time points that each crayfish spent sheltering over the trial period and conducted a Wilcoxon matched pairs test for 27 paired observations. Kendall-Tau tests were performed to assess if the difference in the percentage shelter use of signal and virile crayfish was correlated with differences in chelae length and the number of fights, threats, retreats or avoids performed during interspecific competition trials. To calculate the differences in crayfish shelter use, chelae length and the number of each type of behaviour scored, data for signal crayfish were subtracted from those for virile crayfish. All behaviours were analysed separately.

4.4 Results

4.4.1 *Field survey*

Signal and virile crayfish were captured at 8 and 3 of 19 sites in 2006, and 9 and 10 of 18 sites in 2011, respectively. No sites had both species in 2006, but both species were captured at 5 sites in 2011 (Fig. 4.1). Both signal and virile crayfish expanded their geographical distribution in the study area by approximately 9 km from 2006 to 2011 (Fig. 4.1). Across all sites where crayfish were recovered during 1 or both trapping periods (n = 9 and 8 for signal and virile crayfish respectively), the CPUE of virile crayfish increased significantly from 2006 to 2011 (Wilcoxon matched-pairs test, $V = 0$, $P < 0.01$). Over the same period, the CPUE of signal crayfish did not change ($V = 17$, $P = 0.57$). There was no evidence that the presence of signal crayfish affected the change in virile CPUE between 2006 and 2011; virile CPUE increased at both allopatric ($\bar{x}$ = 4.75 ± 2.06 SE) and sympatric sites ($\bar{x}$ = 2.58 ± 0.94 SE) (Mann-Whitney test, $W = 8$, $P = 1.00$). In contrast, there was evidence that the presence of virile crayfish reduced the CPUE of signal crayfish over the same period: signal CPUE increased at allopatric sites ($\bar{x}$ = 6.42 crayfish ± 2.75 SE) but decreased at sympatric sites ($\bar{x}$ = -0.34 crayfish ± 0.24 SE) ($W = 18$, $P = 0.06$).

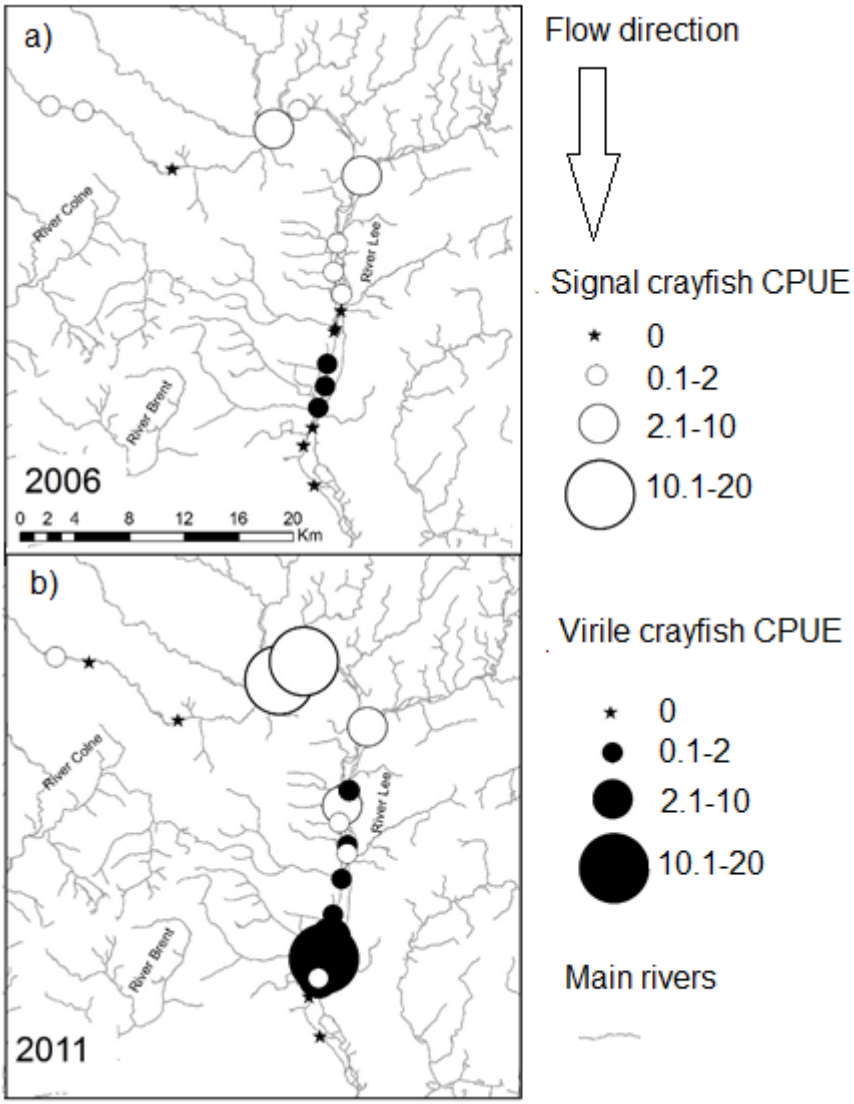

Fig. 4.1. The distribution and catch-per-unit-efforts of signal and virile crayfish at sites along the River Lee, UK, in a) 2006 and b) 2011.

4.4.2 *Shelter preference of individual crayfish*

There was a moderately significant effect of carapace length on shelter use (GLM, $LRT_{1, 58}$ = 3.78, P = 0.05), with smaller crayfish more likely to use shelters. There was no difference in how signal and virile crayfish exploited the 4 refuge types (Fisher's test, P = 0.12), with both species sheltering in the plant pot the most and the artificial plant the least. Signal and virile crayfish did not differentially exploit plastic tubes, the shelter type used in the competition experiment (X^2 = 0.06, df = 1, P = 0.80).

4.4.3 *Competitive interactions*

During dyadic interactions, signal crayfish performed significantly more avoidance (GLM, $LRT_{1, 49}$ = 26.26, P < 0.001) and retreat behaviours ($LRT_{1, 49}$ = 84.71, P < 0.001) and fewer fight

behaviours ($LRT_{1, 49}$ = 90.55, P < 0.001) than virile crayfish (Fig. 4.2). The number of threat behaviours initiated was similar for both species ($LRT_{1, 49}$ = 0.004, P = 0.95) (Fig. 4.2). We did not detect any effects of sex or chelae length on the behavioural interactions of signal and virile crayfish (P > 0.05 for all behaviours).

Virile crayfish spent significantly more time sheltering than signal crayfish (Wilcoxon matched pairs test, V = 333.5, P < 0.001). Differences in the shelter use of the 2 species were not correlated with differences in their chelae size (Kendall Tau, Z = 0.39, P = 0.70). Similarly, the difference in shelter use was not correlated with the difference in the number of fights, threats, retreats, or avoids performed by these crayfish (Kendall Tau, P > 0.05 for all behaviours).

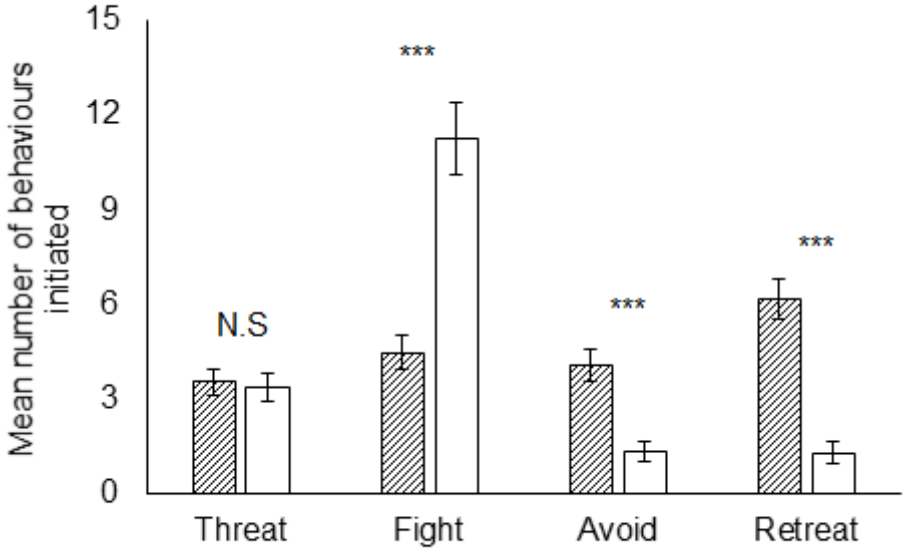

Fig. 4.2. Mean (± SE) number of threat, fight, avoid and retreat behaviours performed by signal (black hatched bars) and virile (white bars) crayfish. *** P < 0.001, N.S not significant for threat behaviours.

4.5 Discussion

Studies combining population field data with laboratory experiments provide mechanistic links between ecological patterns and processes, and are thus critical for understanding interactions between introduced species. Our field observations show that the abundance of newly introduced virile crayfish, but not established invasive signal crayfish, increased significantly over a 5-year period. Comparisons between allopatric and sympatric sites suggest the presence of virile crayfish negatively affected signal crayfish abundance, but not vice versa. Our laboratory experiments showed that virile crayfish were more aggressive than signal crayfish and out-competed them for refugia. Thus, any incumbent advantage of signal crayfish appears

to be outweighed by the competitive superiority of virile crayfish (Duncan and Forsyth 2006; Russell et al. 2014). Given the higher fecundity of virile crayfish (Souty-Grosset et al. 2006), these results suggest that once carrying capacity is reached and resources become limited, virile crayfish may displace signal crayfish through over-invasion and become the dominant crayfish species in the River Lee (Russell et al. 2014).

Over our 5-year field study, both species expanded their range by approximately 9 km, or 1.8 km/year^{-1}. This is similar to the 1.5 km/year^{-1} rate of signal crayfish population spread estimated by Bubb et al. (2004). Unfortunately, no comparable data are available for virile crayfish. These range expansions have resulted in an area of sympatry extending ca. 15 km. Despite small sample sizes, we detected evidence that viriles reduced the abundance of signals in this zone of incipient sympatry. Over the same time the abundance of virile crayfish increased in both allopatric and sympatric sites, albeit at a lower rate in the latter. These data suggest that while both species are negatively affected by competitive interactions, the effect is asymmetric, which is consistent with the balance of empirical studies on interspecific competition (Schoener 1983).

That virile crayfish are superior competitors in laboratory experiments supports our field data conclusion, but is perhaps surprising given the wide range of signal crayfish across Europe (Souty-Grosset et al. 2006) and Japan (Kawai et al. 2004). In comparison, virile crayfish have a smaller invasive range, being restricted to the Netherlands (Souty-Grosset et al. 2006) and our study site in the UK. This difference in invasive range likely results from the widespread use of signal crayfish for aquaculture, and associated introductions across Europe during the 1970s and 80s (Holdich et al. 2014). Despite its currently limited distribution, our results suggest virile crayfish may become widely invasive without effective management interventions.

Our results provide early evidence that biotic resistance and incumbent advantage may not prevent introduced alien species from displacing even notoriously damaging and widespread invasive species (Russell et al. 2014). The likelihood of over-invasion may depend not only on interspecific interactions, but on the propagule pressure of the new alien species (Russell et al. 2014). Unfortunately no data are available on the size of the founder population of virile crayfish in the River Lee, but it is speculated that they originated from exotic pet disposal, and so the founder population is likely to have been relatively small (Ahern et al. 2008). Regardless, minimising virile crayfish propagule pressure in the River Lee is clearly a management priority.

This study highlights the value of combining field data with laboratory experiments to predict how alien species interactions can affect invasion dynamics and the cumulative

ecological impacts of serial invasions. Both signal and virile crayfish are in the spread phase of invasion and regarded as invasive (Blackburn et al. 2011). The management priority for both species is thus containment (Blackburn et al. 2011), which is particularly critical for virile crayfish, which in the UK have only been found in the River Lee. Control programmes for invasive crayfish in this watercourse should, however, carefully consider the effects of 'single' species management on the invasive community (Russell et al. 2014). As the most widely distributed invasive crayfish in the UK, signal crayfish are a management priority (Holdich et al. 2014; Chapter 3). Our results suggest that management interventions targeting signal crayfish in the River Lee could facilitate over-invasion by more aggressive and more fecund virile crayfish. We recommend that control programmes target both species with intensive, sustained trapping by suitably licensed professionals. Any ecological interventions should be complemented by efforts to increase public awareness of the risk of spreading alien crayfish. Both measures should be implemented immediately during the early stages of what may be an over-invasion by virile crayfish (Puth and Post 2005).

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Author contributions

JJ, JRT and JC designed the laboratory study. JJ and JRT conducted laboratory experiments. JJ, JRT, JC, AE and JE designed the field study. JJ, JRT, AE and JE performed the field work. JJ and JRT performed the statistical analysis. AE created the distribution maps. JJ wrote the text. All authors commented on the text.

Chapter 5

The crayfish plague pathogen, *Aphanomyces astaci*, in the UK: distribution and threat to native crayfish

CHAPTER 5: The crayfish plague pathogen, *Aphanomyces astaci*, in the UK: distribution and threat to native crayfish

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5.1 Abstract

Aphanomyces astaci, the causative agent of crayfish plague, has spread throughout Europe, causing a significant decline in native European crayfish species. The introduction and dissemination of this pathogen is attributed to the spread of invasive North American crayfish, which can act as carriers for *A. astaci*. As native European crayfish often succumb to infection with *A. astaci*, determining the prevalence of this pathogen in non-native crayfish is vital to prioritise native crayfish populations for managed translocation. In the current study, 23 populations of invasive signal crayfish (*Pacifastacus leniusculus*) from across England and Wales were tested for *A. astaci* using qPCR. We provide the first molecular evidence of *A. astaci* infecting signal crayfish in the UK, but surprisingly only 56.5% of populations were infected. Furthermore, prevalence within infected sites ranged from just 3.3 to 80%. Microsatellite pathogen genotyping revealed that at least 1 UK signal crayfish population was infected with the group B virulent strain originally isolated from Californian signal crayfish in Sweden. Based on recent crayfish distribution records and the average rate of signal crayfish population dispersal, even with this limited dataset, we identified 1 extant native white clawed crayfish (*Austropotamobius pallipes*) population predicted to come into contact with infected signal crayfish within the next 5 years. This native crayfish population in South Wales should be considered as a priority for translocation. More extensive screening of signal crayfish populations in the UK is needed to assess which other native crayfish populations are at high risk of contracting *A. astaci*.

5.2 Introduction

Crayfish plague, caused by the oomycete *Aphanomyces astaci*, is arguably one of the most deadly invasive parasites of freshwater ecosystems worldwide (Lowe et al. 2004; DAISIE 2009). The pathogen is thought to have been first introduced into Europe (Italy) in 1859, and has subsequently spread throughout most of the continent with the movement of non-native North American (here forth referred to as American) crayfish (reviewed by Alderman 1996; Holdich 2003). Whilst American crayfish are often asymptomatic carriers of the pathogen, in

susceptible native European crayfish infection is usually fatal (Unestam and Weiss 1970; Diéguez-Uribeondo et al. 1997; Bohman et al. 2006; Kozubíková et al. 2008, Oidtmann 2012). Therefore monitoring the global spread of this pathogen is a conservation priority.

One of the main American crayfish species responsible from spreading *A. astaci* in Europe, the signal crayfish (*Pacifastacus lenisculus*) was first introduced into the UK from Sweden during the 1970s for aquaculture (e.g. Holdich and Reeve 1991; Alderman 1996; Peay and Hiley, 2005; Holdich et al. 1999, 2014). This corresponded with mass declines in Britain's historically abundant native white clawed (*Austropotamobius pallipes*) crayfish (Holdich and Reeve 1991; Holdich et al. 2009; Holdich and Sibley 2009; Holdich et al. 2014; Chapter 3), to such an extent that since 2010 they have been categorised as endangered (IUCN 2015). Whilst it was widely considered that reductions in native crayfish were, at least partially, due to the transmission of *A. astaci* from signal crayfish, this pathogen was not detected in the UK until the early 1980s (Alderman 1996; Holdich 2003). One of the first suspected outbreaks of plague in the UK was recorded from the River Lee, Thames catchment, England in 1981 (Alderman 1996; Holdich 2003). The pathogen has since been reported in native crayfish from several other sites in England as well as Wales and Ireland (Alderman et al. 1984, 1990; Holdich and Reeve 1991; Alderman 1996; Lilley et al. 1997; Holdich 2003). These previous reports, however, have been based on pathogen morphology and native host symptoms. Given that there are no morphological features that distinguish *A. astaci* from non-pathogenic *Aphanomyces* species (Royo et al. 2004; Oidtmann 2012), molecular confirmation is essential (Oidtmann et al. 2006; Vrålstad et al. 2009). The only molecular report of *A. astaci* in the UK is actually from another introduced crayfish species (*Orconectes* cf. *virilis*) (see Tilmans et al. 2014), which is restricted to a single catchment (Chapter 4).

Gaining a comprehensive understanding of *A. astaci* distribution in the UK is essential for native crayfish conservation. It is generally considered that the only way of ensuring the sustainability of white clawed crayfish in the UK is through the establishment of isolated "ark sites" free from non-native crayfish and at low risk of their invasion (Peay 2009). Resources for implementing such conservation measures are, however, limited and so the selection of native crayfish populations for translocation needs to be a well-informed process. Native crayfish populations in close vicinity to *A. astaci*-infected invasive crayfish populations are at higher risk of extirpation than those neighbouring uninfected ones (Söderbäck 1994; Westman et al. 2002; Schulz et al. 2006; Dunn et al. 2009; Schrimpf et al. 2013). Native crayfish are in fact capable of co-existing with invasive crayfish for several years in the absence of *A. astaci* (see Söderbäck 1994; Westman et al. 2002; Schulz et al. 2006; Dunn et al. 2009; Schrimpf et

al. 2013). It is therefore, arguably, of greater urgency to translocate native crayfish populations at high risk of *A. astaci* transmission, than those in close proximity to uninfected invasive crayfish.

Here, we used qPCR to assess the prevalence and intensity of infection with *A. astaci* in 23 populations of invasive signal crayfish in England and Wales. Using these data in combination with long term white clawed crayfish distribution records (see Chapter 3) we identified native crayfish populations at high risk of infection with *A. astaci* (determined by their proximity to an *A. astaci*-infected signal crayfish population). Given that *A. astaci* genotypes differ in virulence (Makkonen et al. 2012), when possible, we also genotyped the strain of *A. astaci*.

5.3 Materials and Methods

For this study, invasive signal crayfish (*Pacifastacus leniusculus*) from the UK were screened for the presence of *Aphanomyces astaci* using similar molecular methods in 2 separate laboratories; the Centre for Environment, Fisheries and Aquaculture Science, UK (Cefas), and Charles University in Prague, Czech Republic (CUP). Crayfish processed at Cefas were collected between September 2009 and July 2010 from 17 sites across England and Wales (*n* = between 8 and 30 animals per site, Table 5.1). Upon collection, animals were transported to the Cefas laboratory in Weymouth and humanely euthanized by exposure to chloroform vapours. Crayfish analysed at CUP were harvested from 6 sites in England and Wales during May-September, 2014 (*n* = 20-30 animals per sites, Table 5.1). At all of these sites, signal crayfish were collected. Prior to transportation to CUP these animals were humanely euthanized by freezing at -80^{o}C in Cardiff University and packaged individually in falcon tubes containing 95% molecular grade ethanol. At all sites crayfish were captured using baited traps.

From each crayfish, a section of tail fan and soft abdominal cuticle were harvested for *A. astaci* screening. For animals processed in CUP soft cuticle from 2 limb joints and any sections of melanised cuticle were also collected and pooled (Svoboda et al. 2014). At Cefas tissue samples from the tail fan and soft abdominal cuticle were analysed separately (mean: 60 and 78 mg of tissue per host sample for the tail fan and soft abdominal cuticle respectively). For these samples, tissue disruption was conducted in fast prep tubes containing lysis matrix A (MP biomedicals) and DNA subsequently extracted using the Qiamp DNeasy Biorobot investigator kit (Qiagen), according to the manufacturer's guidelines. At CUP, for each animal, all collected tissue samples were amassed (40-50 mg per host sample) and ground together in

liquid nitrogen. DNA was then extracted using DNeasy tissue kit (Qiagen) in accordance with manufacturer's instructions.

All samples were tested for *A. astaci* presence with the TaqMan MGB quantitative PCR (qPCR) as described in Vrålstad *et al.* (2009); with a slightly altered protocol (in accordance with Tuffs and Oidtmann, 2011 at Cefas, and Strand et al. 2011; Svoboda et al. 2014 at CUP) to increase assay specificity. At Cefas and CUP qPCRs were run on a Step one Plus real time cycler (Applied Biosystems) and an iQ5 BioRad thermal cycler, respectively. Negative controls were used in every step of the procedure; these remained negative in all cases. Based on the strength of the PCR signal, we assigned the relative level of *A. astaci* infection to semi-quantitative agent levels (A0-A7; according to Vrålstad et al. 2009; Kozubíková et al. 2011). Samples designated as A2 or higher were considered positive for *A. astaci* presence.

For *A. astaci* genotype group identification, an *A. astaci*-positive signal crayfish (harbouring an A3 agent level infection) from the Mochdre Brook (Wales) was analysed using 9 *A. astaci*-specific microsatellite markers (Grandjean et al. 2014) at CUP. Prior to genotyping the sample was concentrated using a Concentrator Plus 5305 (Eppendorf). The results were compared with the *A. astaci* reference strains described in Grandjean et al. (2014).

As we were only able to test a fraction of the signal crayfish populations in the UK (see Chapter 4 for detailed distribution information) for *A. astaci*, comprehensively assessing the risk this pathogen poses native crayfish in the UK was beyond the scope of the current study. Nevertheless, we assessed native white clawed crayfish populations at potential risk from the 13 signal crayfish populations where we detected *A. astaci* using recent (2009 onwards) native crayfish distribution records. Sites where *A. astaci* was detected were mapped and any native crayfish populations within a 7.5, 10, 12 or 15 km radius, as the crow flies, recorded. Buffer zones (i.e. 7.5, 10, 12 and 15 km) were selected on the basis that the average rate of signal crayfish population expansion along a river is 1.5 km per year (Bubb et al. 2004). Therefore populations within 7.5 km of each other are predicted to come into contact within 5 years, providing that they inhabit connected waterbodies These analyses were performed using ArcGIS version 10.3 mapping software.

5.4 Results

Aphanomyces astaci was detected in 56.5% (13 out of 23) signal crayfish sites in Wales and England (Table 5.1, Fig. 5.1). Among infected populations, prevalence ranged from 3.3-80% with generally low infection intensities (agent levels A2-A3) with the exception of Mochdre Brook in Wales, and Bently Brook and River Lee in England (Table 5.1, Fig. 5.1). A multilocus

microsatellite genotype was only obtained from the Mochdre Brook population. This was identical to the reference axenic culture of the genotype group B at 8 loci, but was homozygote rather than heterozygote at the Aast9 locus (Table 5.2).

Table 5.1. Prevalence and infection intensity of *Aphanomyces astaci* in British invasive signal crayfish (*Pacifastacus leniusculus*) populations. Infection intensities are reported as semi-quantitative agent levels (Vrålstad et al. 2009): uninfected (A0-1) and infected (A2-5). Animals from sites marked with an † were processed in Charles University (Prague), all other samples were analysed at the Centre for Environment, Fisheries and Aquaculture Science, Weymouth (UK).

Population (location)	**NGR (approx.)**	**Prevalence (%)**	**No. animals tested (n)**	**Agent level (range)**
Wales				
†Sirhowy River (Caerphilly)	ST178961	0	30	A0
†Dderw farm pond (Powys)	SO138375	0	30	A0-A1
†Bachowey River 1 (Powys)	SO168457	50	20	A0-A3
Bachowey River 2 (Powys)	SO158464	23.3	30	A0-A3
†Gavenny River (Monmouthshire)	SO310163	46.7	30	A0-A3
†Mochdre Brook (Powys)	SO086904	75	20	A0-A4
England				
Broadmead Brook (Wiltshire)	ST822768	0	30	A0
St Catherine's Brook (South Gloucestershire)	ST786705	0	30	A0
Sutton Bingham Reservoir (Somerset)	ST555114	0	30	A0
River Wharfe 1 (North Yorkshire)	SD978674	0	30	A0
River Riddle (Cumbria)	SD593841	0	30	A0
Fenny Beck (West Yorkshire)	SE174171	0	29	A0
Great Ouse (Suffolk)	TL725739	0	30	A0
†River Lugg (Herefordshire)	SO522523	0	30	A0-A1
Tetbury Avon (Wiltshire)	ST923881	3.3	30	A0-A3
River Hamps (Staffordshire)	SK063538	20	10	A0-A3
River Wharfe 2 (North Yorkshire)	SE000633	37.5	8	A0-A3
River Evenlode (Oxfordshire)	SP439117	27.6	29	A0-A3
River Thame (Aylesbury)	SP677066	80	30	A0-A3
River Wid (Norfolk)	TL666988	18.5	27	A0-A3
River Ash (Hertfordshire)	TL384142	6.7	30	A0-A3
River Lee (Hertfordshire)	TL328130	16.7	30	A0-A4
Bently Brook (Derbyshire)	SK177483	10	30	A0-A5

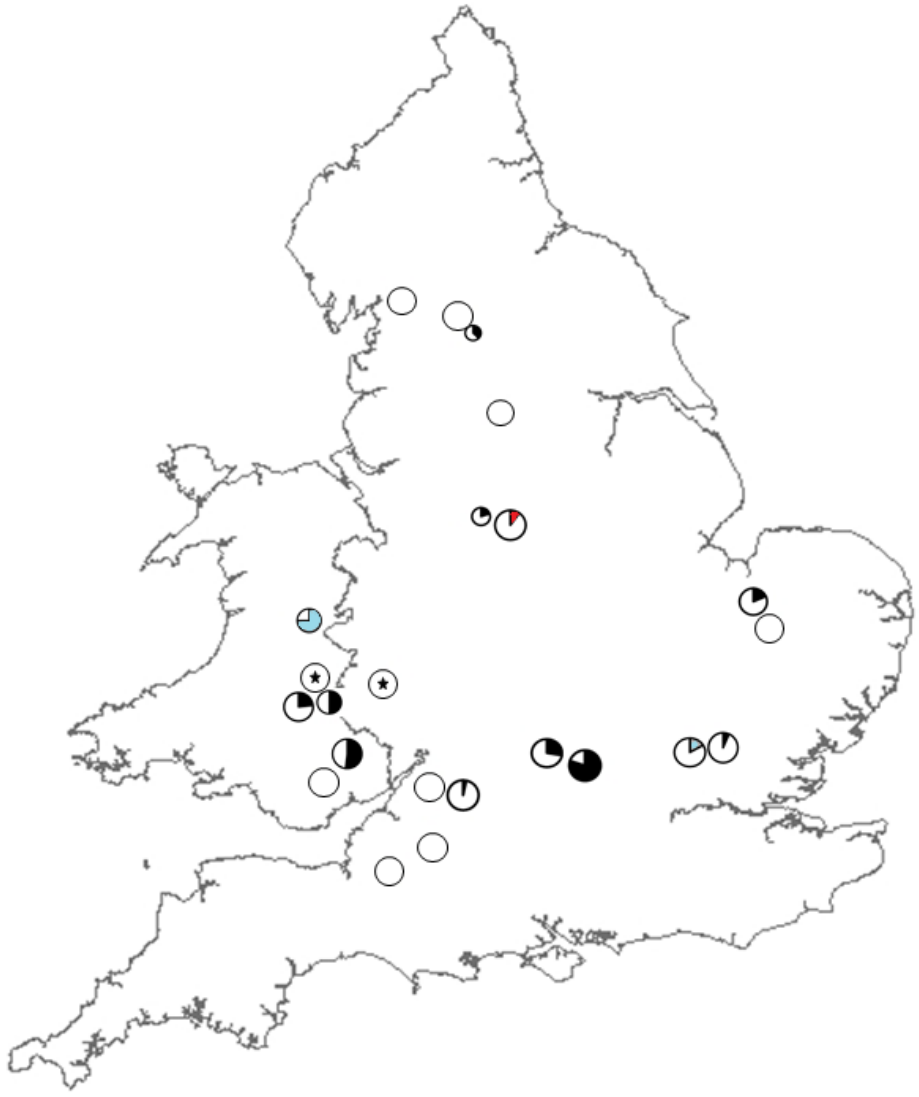

Fig. 5.1. Location of the invasive signal crayfish populations tested for *Aphanomyces astaci* in the current study using qPCR. For each population, the percentage of crayfish tested that were infected with *A. astaci* (i.e. the pathogen prevalence) is shown using a pie chart, with the shaded portion of each chart representing infected individuals, and the diameter of the circle the sample size (n = 8-30). Black shading indicates that the highest infection intensity (reported as semi-quantitative agent levels, see Vrålstad et al. 2009) detected was A3, blue A4 and red A5. White circles show populations where the pathogen was not detected at any level (A0). Circles containing black stars represent those populations where trace levels of the pathogen (A1) were amplified. As an infection intensity of A1 is below the limit of detection for the method used these populations are classed as uninfected.

In total 15 native crayfish populations (confirmed extant at some time point between 2009 and 2014) were found to be within 15 km of an *A. astaci* infected signal crayfish population (Table 5.3). Of these, the population in River Cilcenni, South Wales, was closest (within 7.5 km) to infected signal crayfish (Table 5.3). These infected crayfish from the Bachowey River were also within 15 km of an additional 6 extant native crayfish populations (Table 5.3).

Table 5.2. Comparison of allele sizes of 9 microsatellite loci from the reference strains of *Aphanomyces astaci* genotype group B (Grandjean et al. 2014) and an *A. astaci*-positive signal crayfish (*Pacifastacus leniusculus*) from a UK population (Mochdre Brook).

Locus	Reference sequence	UK population
Aast 2	142	142
Aast 4	87	87
Aast 6	148	148
Aast 7	215	215
Aast 9	164/182	164
Aast 10	132	132
Aast 12	226/240	226/240
Aast 13	202	202
Aast 14	248	248

Table 5.3. Location and year of most recent record of native white clawed crayfish (*Austropotamobius pallipes*) populations (data from CrayBase: Chapter 3) in close vicinity to *Aphanomyces astaci* infected invasive signal crayfish (*Pacifastacus leniusculus*). For each native crayfish population information is also provided on the location and proximity of the nearest *A. astaci* infected signal crayfish population.

White clawed crayfish			Signal crayfish	
Population	**Location (country)**	**Most recent record**	**Population(s)**	**Proximity (km)**
Cilcenni	Wales	2009	Bachowey River 1 and	7.5
Scithwen	Wales	2014	Bachowey River 1 and	10.5
Clettwr	Wales	2014	Bachowey River 1 and	10.5
Rhiwiau Brook	Wales	2009	Bachowey River 1 and	12
Llynfi Dulas	Wales	2014	Bachowey River 1 and	15
River Ennig	Wales	2011	Bachowey River 1 and	15
Cwm Sheppard	Wales	2010	Bachowey River 1 and	15
Nant Onnau	Wales	2010	Gavenny River	10.5
Dulas Brook	Wales	2009	Gavenny River	15
Marden	England	2009	Tetbury Avon	15
By brook	England	2009	Tetbury Avon	15
Lurscombe	England	2009	Tetbury Avon	15
Winterburn beck	England	2010	River Wharfe 2	10.5
River Wharfe	England	2009	River Wharfe 2	15
Swinbrook	England	2010	River Evenlode	15

5.5 Discussion

The current study provides the first molecular confirmation of *Aphanomyces astaci*, the causative agent of crayfish plague, in invasive signal crayfish (*Pacifastacus leniusculus*) from

the UK. Whilst this seemingly affirms the perceived role of *A. astaci* causing native crayfish declines (Holdich 2003), not all signal crayfish populations tested were infected. In fact, *A. astaci* was only found in just over half (56.5%) of UK signal crayfish populations, and within these the prevalence varied between 3.3 and 80%. This contradicts the traditional assumption in the UK that all American crayfish populations are carriers of *A. astaci* (see Cerenius et al. 2003), but this is not without precedence. Recently, *A. astaci*-uninfected American crayfish have been detected in other European countries (Kozubíková et al. 2009; Skov et al. 2011; Filipová et al. 2013).

In the current study, microsatellite genotyping revealed the presence of an *A. astaci*-positive DNA isolate apparently belonging to genotype group B in signal crayfish from Mochdre Brook (Wales). This is perhaps unsurprising given that, within Europe, group B strains of *A. astaci* were first isolated from invasive signal crayfish in Sweden (Huang et al. 1995), which is considered as the country of origin for most signal crayfish introduced into the UK during the 1970s and 80s (Holdich et al. 1999). Isolation of this highly virulent strain of *A. astaci* (see Makkonen et al. 2012) may explain the mass mortalities of native white clawed in the UK following the introduction of signal crayfish (see Chapter 3). Although chronic *A. astaci* infections have been observed in other native European crayfish (e.g. Jussila et al. 2011; Kokko et al. 2012; Pârvalescu et al. 2011; Schrimpf et al. 2012; Kušar et al. 2013), these may be caused by the less pathogen group A 'old' strain (Makkonen et al. 2012). Therefore, ideally information on *A. astaci* strain should be considered when assessing the risk posed to native crayfish. Although, further studies are still required to determine the virulence of others strains.

Given that the conservation of native crayfish in the UK is generally considered to be dependent upon the translocation of animals into "ark sites" (Peay 2009), and that resources for implementing such measures are limited, targeting removal of native crayfish populations at the greatest risk of extirpation is critical. Native European crayfish can co-exist with American crayfish for extended periods of up to 30 years in the absence of *A. astaci* (see Schulz et al. 2006; Dunn et al. 2009; Schrimpf et al. 2013; Skov et al. 2011; Westman et al. 2002), but are often rapidly extirpated if this pathogen is present (e.g. Holdich and Reeve 1991; Vennerström et al. 1998; Bohman et al. 2006; Kozubiková et al. 2008). Therefore, native white clawed crayfish populations in close vicinity to *A. astaci*-infected signal crayfish are predicted to be at greater risk of local extinction than those neighbouring uninfected signal crayfish.

Considering that only a portion of the signal crayfish populations existing in the UK were screened in the current study, and of these only around half were infected with *A. astaci*, increased testing for this pathogen is needed to comprehensively assess native crayfish

populations at greatest risk of disease transmission. Nevertheless, for the 13 signal crayfish populations where we detected *A. astaci* we identified 1 white clawed crayfish population recorded since 2009, located within 7.5 km. This native crayfish population inhabits the Cilcenni within the Wye catchment, South Wales, and was most recently detected in 2009. Given its proximity to infected signal crayfish, we recommend that this population is considered as a priority for translocation into an "ark site," although we acknowledge that increased screening of signal crayfish for *A. astaci* may reveal other native crayfish populations at greater risk of extirpation. Determining the exact order of translocation priority for the 14 native crayfish populations within 7.5 and 15 km of an *A. astaci* infected signal crayfish population is beyond the scope of the current study. For extant populations factors that should be considered when assessing translocation priority include; proximity to infected crayfish, connectivity of water bodies housing native and infected invasive crayfish, prevalence of *A. astaci* in the nearest infected crayfish population, density of crayfish present in the native crayfish and neighbouring infected signal crayfish population, and whether any barriers in the environment exist that may prevent animals from either population dispersing. Additionally, as native crayfish populations can be rapidly extirpated by crayfish plague, surveying to confirm the persistence of populations under consideration for translocation should always be a pre-requisite.

Whilst the current study confirms that signal crayfish from England and Wales are infected with *A. astaci*, pathogen presence and prevalence varied between populations. Therefore, there is a danger, in terms of assessing risk to native crayfish, of assuming that all invasive American crayfish populations in the UK are infected with this pathogen. Based on our findings we recommend increased *A. astaci* screening, using appropriate pathogen specific molecular methods, of signal crayfish populations in the UK, to fully assess the risks to native crayfish and target populations for translocation.

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Author contributions

JJ, JC, AP, AM, SNT and BO designed the study. JJ, AM, NVR and SNT performed the genetic tests. JJ conducted the field work. JJ and AM performed the statistical analyses. JJ created the risk map. JJ wrote the text. All authors commented on the text.

Chapter 6

Aparent transmission of *Aphanomyces astaci* from invasive signal to virile crayfish in the UK

CHAPTER 6: Aparent transmission of *Aphanomyces astaci* from invasive signal to virile crayfish in the UK

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6.1 Abstract

The crayfish plague pathogen (*Aphanomyces astaci*) causes mass mortalities of European crayfish when transmitted from its original North American crayfish hosts. Little is known, however, about its interspecific transmission between different American crayfish. We screened signal and virile crayfish inhabiting a UK river for *A. astaci* infection at allopatric and sympatric sites. Signal crayfish were infected at both sites, virile crayfish only in sympatry. Genotyping of *A. astaci* from virile crayfish suggested the presence of a strain related to one infecting British signal crayfish. We conclude that virile crayfish likely contracted *A. astaci* interspecifically, upon contact with infected signal crayfish.

6.2 Introduction

The crayfish plague agent, the oomycete *Aphanomyces astaci*, is arguably one of the most devastating invasive parasites of European freshwaters (Lowe 2004; DAISIE 2009). Since its first introduction in the mid-19th century (Alderman 1996; Holdich 2003), the pathogen has spread throughout Europe, largely through movements of non-native North American (here forth referred to as American) crayfish (Souty-Grosset et al. 2006; Holdich et al. 2014; Chapter 3). Whilst American crayfish are often asymptomatic of *A. astaci* infection, the disease is usually lethal in European species (Unestam and Weiss 1970; Diéguez-Uribeondo et al. 1997; Bohman et al. 2006; Kozubíková et al. 2008). Once introduced, crayfish plague can spread rapidly, transmitted through zoospores that are released into the water (Oidtmann et al. 2002) and can survive for at least 14 days (CEFAS 2000). Spores are mainly released during host moulting or death (Oidtmann et al. 2002; Svoboda et al. 2013, but see Strand et al. 2012), and within a cadaver *A. astaci* can remain viable for several days (Oidtmann et al. 2002). Therefore, the movement of infected carcasses by predators could facilitate pathogen dispersal. If fish ingest infected tissue, the pathogen can even survive passage through the gastro-intestinal tract, providing an additional transmission pathway (Oidtmann et al. 2002).

Whilst the transmission of crayfish plague pathogen from non-native American to European crayfish has been widely documented (e.g. Alderman et al. 1990; Diéguez-Uribeondo

et al. 1997; Vennerström et al. 1998; Bohman et al. 2006), little is known about interspecific transmission between these invasive carriers. Until now, 4 different *A. astaci* genotype groups have been isolated in Europe; group A was obtained from infected native European crayfish (*Astacus astacus* and *A. leptodactylus*) and groups B, D and E from different American crayfish (*Pacifastacus leniusculus*, *Procambarus clarkii* and *Orconectes limosus*, respectively) (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011). The genotype groups infecting additional *A. astaci* carriers known from European waters, calico (*Orconectes immunis*), marbled (*Procambarus fallax* f. *virginalis*) and virile (*Orconectes* cf. *virilis*) crayfish (Schrimpf et al. 2013; Tilmans et al. 2014; Keller et al. 2014), are so far unknown. Existing data suggest that *A. astaci* genotype groups are host-specific among American crayfish (Grandjean et al. 2014). There is no evidence of strains transmitting between these crayfish in the wild, although it seems to occur in the aquarium trade (Mrugała et al. 2015).

Here, we investigate interspecific transmission of *A. astaci* upon contact of 2 potential carrier species. We screened non-native signal (*P. leniusculus*) and virile (*O.* cf. *virilis*; see Filipová et al. 2010) crayfish from allopatric and sympatric sites within the River Lee and an adjacent lake, London (UK) for the presence of *A. astaci*, and, where possible, genotyped infected host specimens. Signal crayfish were initially stocked in this river during the mid-1970s (Almeida et al. 2014), whereas virile crayfish were unintentionally introduced there around 2004 (Ahern et al. 2008). The 2 species have been co-existing since at least 2011 (Chapter 4). Virile crayfish in this river have already been reported to carry *A. astaci* (see Tilmans et al. 2014). Our aim was to elucidate whether virile crayfish in the UK are infected with a distinct strain or one which has been contracted from co-existing signal crayfish.

6.3 Methods

Invasive signal crayfish and virile crayfish were collected from the River Lee and an adjacent lake, London, UK, during September 2014. Using baited traps employed over 2 consecutive nights and checked daily, animals were caught from allopatric (National Grid Reference: TL386082, TL368029 for signal and virile crayfish respectively, $n = 30$ for each species) and sympatric sites (National Grid Reference: TL370027, $n = 9$ signal and 30 virile crayfish). Upon capture, animals were transported individually to Cardiff University (UK), humanely euthanized by freezing at -80ºC and stored in ca. 95% molecular grade ethanol before transport to Charles University in Prague for further processing. For *A. astaci* screening we harvested, from each animal, a section of tail fan, soft abdominal cuticle, 2 limb joints, and any melanised cuticle (as in Svoboda et al. 2014). Tissue samples from each individual (40-50 mg) were

ground together in liquid nitrogen from which DNA was extracted using a DNeasy tissue kit (Qiagen) as per manufacturer's guidelines.

All samples were screened for *A. astaci* presence using TaqMan MGB quantitative PCR (qPCR) on the iQ5 BioRad thermal cycler according to Vrålstad et al. (2009), slightly modified to increase assay specificity (Strand et al. 2011; Svoboda et al. 2014). To check for potential inhibition (as per Kozubíková et al. 2011; Svoboda et al. 2014), DNA isolates were included in 2 concentrations (undiluted and 1:10 dilution) for each qPCR. In each step of the protocol negative controls were used, and in all cases these remained negative. Based on the strength of the PCR signal, we designated the relative level of *A. astaci* infection to semi-quantitative agent levels (A0-A7; according to Vrålstad et al. 2009; Kozubíková et al. 2011). Samples with agent levels of A2 or higher were considered positive for *A. astaci*.

For *A. astaci* genotype group identification, *A. astaci*-positive samples were analysed using 9 *A. astaci*-specific microsatellite markers (Grandjean et al. 2014). As amplification success depends on the amount of pathogen DNA in the sample, genotyping was only performed for those with agent level A3 and higher (as in Grandjean et al. 2014). In case of an initial lack of amplification, DNA isolates were concentrated on the Concentrator Plus 5305 (Eppendorf). The results were compared with the *A. astaci* reference strains described in Grandjean et al. (2014) and an *A. astaci*-positive DNA isolate from signal crayfish in Lake Mochdre (Newtown) Wales, UK.

6.4 Results

Within allopatric sites on the River Lee, *Aphanomyces astaci* was detected in 83% (25 out of 30) signal crayfish but was not detected in any virile crayfish (n = 30). From the sympatric site, 44% (4 out of 9) signal crayfish and 23% (7 out of 30) virile crayfish tested positive for crayfish plague infection. All *A. astaci*-positive samples yielded low levels of infection (A2-A3; Vrålstad et al. 2009).

Due to low amount of *A. astaci* DNA, the reliable amplification and scoring of the microsatellites were only possible for 1 specimen of the virile crayfish; for 7 out of the 9 microsatellite loci. The multilocus genotype corresponded at 5 loci to the reference axenic culture of the genotype group B (Table 6.1). The differences were observed at the Aast9 and Aast12 loci, where homozygotes rather than heterozygotes were scored. Such variation at the Aast9 locus has been also observed in the *A. astaci*-positive DNA isolate from signal crayfish in Wales, UK (Table 6.1).

Table 6.1. Comparison of allele sizes of 9 microsatellite loci from the reference strains of *Aphanomyces astaci* genotype group B (Grandjean et al. 2014) and *A. astaci*-positive samples of *Orconectes* cf. *virilis* and *Pacifastacus leniusculus* from Wales, UK. n/a – loci with no amplification, likely due to low concentration of *A. astaci* DNA in the isolates.

Locus	Reference sequence (*P. leniusculus*)	UK population (*P. leniusculus*)	UK population (*O. cf. virilis*)
Aast 2	142	142	142
Aast 4	87	87	87
Aast 6	148	148	n/a
Aast 7	215	215	215
Aast 9	164/182	164	164
Aast 10	132	132	n/a
Aast 12	226/240	226/240	240
Aast 13	202	202	202
Aast 14	248	248	248

6.5 Discussion

Here we present evidence of interspecific transmission of *Aphanomyces astaci* between 2 American crayfish species invasive in the UK. Virile crayfish were infected at the site where they coexisted in the River Lee with signal crayfish, but not at the allopatric site. In contrast, signal crayfish were infected with *A. astaci* at both sympatric and allopatric sites. The *A. astaci* genotype identified in virile crayfish was similar, but not identical, to the reference strain of the genotype group B, isolated in Europe from infected signal crayfish (Huang et al. 1994; Grandjean et al. 2014) and to an isolate from a UK signal crayfish population (Table 6.1). Such intra-genotype group variation has been reported previously (Grandjean et al. 2014). Although Tilmans et al. (2014) speculated that virile crayfish were already infected by *A. astaci* prior to their introduction to European waters, our results suggests that those in the UK have contracted the pathogen through interspecific transmission from co-existing signal crayfish. Such interspecific transfer between *A. astaci* carriers has not previously been documented in the wild.

Possessing a wide host range is one of the key factors in determining the success of an introduced parasite (Kennedy 1994). Therefore, the ability of *A. astaci* to transmit between American carrier species has likely facilitated the invasion success of this pathogen in those countries harbouring multiple American crayfish species (Souty-Grosset et al. 2006; Kouba et al. 2014; Chapter 3). From a conservation perspective this is particularly concerning considering the lethality of the disease to native European crayfish (Unestam and Weiss 1970; Alderman et al. 1990; Diéguez-Uribeondo et al. 1997; Vennerström et al. 1998; Bohman et al. 2006), of which the white clawed (*Austropotamobius pallipes*) and noble (*Astacus astacus*) crayfish are respectively designated as endangered and vulnerable by the IUCN (2015).

The likely ability of crayfish plague strains to transmit between different American carrier species is particularly important given that genotypes vary in virulence (Makkonen et al. 2012, 2014; Viljamaa-Dirks et al. 2013) and/or climate requirements (Diéguez-Uribeondo et al. 1995; Rezinciuc et al. 2014). We suggest that within a given region all species of American crayfish should be considered as potential vectors of crayfish plague, until they are confirmed otherwise (see Tilmans et al. 2014). Increased effort should be focussed into genotyping crayfish plague to monitor the spread of infection, and prioritise protection of native populations threatened by the more virulent strains of the pathogen.

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Author contributions

JJ, JC, AP and AM designed the study. JJ and AM performed the genetic tests. JJ conducted the fieldwork. JJ and AM performed the statistical analyses. JJ wrote the text. All authors commented on the text.

Chapter 7

Two alien species of Branchiobdellida (Annelida: Clitellata) new to the British Isles: a morphological and molecular study

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Research Article

Two alien species of Branchiobdellida (Annelida: Clitellata) new to the British Isles: a morphological and molecular study

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7.1 Abstract

Freshwater ecosystems are particularly vulnerable to the effects of alien species and decapod crustaceans, notably crayfish, are a principal threat. Although symbiotic fauna may influence the impact and dispersal of introduced species, this is often overlooked. Here we provide the first record of non-native ectosymbiotic branchiobdellidan worms on invasive signal crayfish (*Pacifastacus leniusculus* Dana 1852) in the British Isles. Using morphological and molecular techniques we identified and re-described 2 branchiobdellidan species new to the UK, *Xironogiton victoriensis* Gelder and Hall 1990 and *Cambarincola* aff. *okadai* Yamaguchi, 1933, both of which were found at a single location in the Gavenny River, South Wales. The prevalence of *X. victoriensis* and *C.* aff. *okadai* was 75.34% and 71.23% respectively. Although the level of *X. victoriensis* and *C.* aff. *okadai* co-infection was high at 75.41% of all infected animals, the 2 species exhibited different micro-habitat preferences on the host with the former being found predominantly on the chelae and walking legs and the latter on the carapace and abdomen. For both branchiobdellidan species, worm burdens were positively correlated with crayfish size. The lack of branchiobdellidan records from signal crayfish in nearby water bodies, and the reports of native white clawed crayfish (*Austropotamobius pallipes*) in the Gavenny as recently as 2000, indicates that introduction of this worm infested population occurred relatively recently, despite stringent legislation banning the import and transportation of non-native crayfish into the UK.

7.2 Introduction

Invasive species are a principal threat to global biodiversity (Wilcove et al. 1998), but it is often overlooked that their dispersal and impact on native biota may be influenced by symbionts

(Torchin et al. 2002). North American crayfish species are among the most successful and widespread invasive species whose effects on native crayfish are exacerbated when infected with *Aphanomyces astaci* (Schikora 1906), the causative agent of crayfish plague (Unestam and Weiss 1970; Holdich and Reeve 1991; Kozubíková et al. 2009; Schrimpf et al. 2013). Whilst North American crayfish are largely resistant to this parasite, they act as reservoirs and vectors of the disease, increasing its transmission to susceptible native European crayfish in which infection is reportedly always lethal (Unestam and Weiss 1970). Whilst the majority of studies on crayfish symbionts are focused on *A. astaci*, crayfish are host to several other fungi, viruses, bacteria, protists, helminths and annelids (reviewed by Longshaw 2011). One group of organisms that are frequently introduced on invasive crayfish are branchiobdellidan worms (Gelder 1996). These ectosymbiotic annelids live primarily on astacoidean crayfish (Govedich et al. 2009) and are considered obligate ectosymbionts, as reportedly their cocoons only embryonate if attached to a live host (Govedich et al. 2009).

The relationship between crayfish and branchiobdellidans can vary across the symbiosis continuum from mutualism (e.g. Brown et al. 2002, 2012; Lee et al. 2009) to commensalism (e.g. Bishop 1968; Keller 1992; Govedich et al. 2009) and parasitism (Vogt 1999; Brown et al. 2012; Rosewarne et al. 2012) depending on host, branchiobdellidan species and density, and environmental conditions. Branchiobdellidans, therefore, have the potential to affect the invasion success of crayfish either facilitatively or detrimentally. Despite this branchiobdellidans are relatively understudied and their distribution on invasive crayfish in the British Isles has not been assessed. To our knowledge there are only 3 previous reports of branchiobdellidans (*Branchiobdella astaci* Odier 1823) in the UK (Leake and Price 1965; Rogers et al. 2003; Rosewarne et al. 2012), all on native white clawed crayfish (*Austropotamobius pallipes* Lereboullet 1858).

Here we surveyed 17 sites for signal crayfish (*Pacifastacus leniusculus*) in Wales and bordering parts of England (Herefordshire) of which one contained crayfish infected with branchiobdellidans. Using a combination of morphological and molecular techniques we identified 2 species of branchiobdellidans in Wales, *Xironogiton victoriensis* (Gelder and Hall 1990) and an unknown species putatively identified as *Cambarincola* aff. *okadai* that is morphologically similar to, but genetically distinct from, *C. okadai* (Yamaguchi 1933). This is the first know record of branchiobdellidans on invasive crayfish in the UK. From field survey data we examined the prevalence, mean intensity and micro-habitat use of these branchiobdellidans on the crayfish host. We present detailed morphological descriptions of both species that can be compared against alien branchiobdellidans subsequently found in the UK or

mainland Europe to help monitor non-native species' movements. Our species descriptions will also assist in the future identification of *C.* aff. *okadai*.

7.3 Methods

7.3.1 *Field surveys*

The UK national crayfish database, CrayBase (Chapter 3), was used to identify sites positive for signal crayfish populations in Wales and bordering Herefordshire (England) between 1975 and 2012. Each of the 17 sites was surveyed once during July-October 2012 (Table 7.1), using either standard stone turning and kick sampling protocols (n = 15 sites) or baited traps (n = 2 sites) left to fish overnight, where manual surveying was unsuitable. Upon capture, the external surfaces of all crayfish were examined for branchiobellidans. All crayfish were sexed, measured (carapace length; mm) and any signs of disease recorded. Additionally we noted if crayfish were in inter-moult (carapace hard), pre/post-moult (carapace hard but could be depressed) or moult (carapace completely soft) condition. As pre and post-moult crayfish are difficult to definitively discriminate these categories were combined.

At the site where branchiobdellidans were located in 2012 an additional 6 manual surveys were conducted (1 in Oct 2012, and 5 between April and June 2013) to determine the prevalence, mean intensity and distribution of worms on the crayfish. A sub-sample of worms (n = 30, Oct 2012 collection) were carefully removed from the external surface of the crayfish (n = 2) using forceps and preserved in 90% molecular grade ethanol for subsequent identification. Analysis of these branchiobdellidans indicated the presence of at least 2 branchiobdellidan species. Therefore for subsequent surveys conducted in 2013, all worms were removed from the external surface of the crayfish host (their position noted) and stored separately in 90% ethanol according to the location on the crayfish from which they were removed. Furthermore, a subset of crayfish (n = 10) were dissected to examine the gill tissue for branchiobdellidans.

7.3.2 *Morphological identification*

Branchiobdellidans were examined live in the laboratory, narcotized by the gradual addition of 7% $MgCl_2$ (later fixed in 4% formalin), or preserved in 100% ethanol. Some specimens were stained temporarily with alcohol solutions of Methyl Green (e.g., Mackie and Gobin 1993) or Shirlastain A (Petersen 1998) to aid observation of the morphological characteristics. Internal features were examined following dissection.

Photographs of live worms were taken using Live View image capture in Manual mode with mirror lock-up enabled (Canon EOS Utility 2.10.4; Apple Intel Core 2 Duo MacBook Pro) via a stand-mounted Canon EOS 550D camera, fitted with an EFS 60mm or MP-E 65mm (1-5x) macro lens and a cable mounted Speedlight 580EX II Flash. Raw images were edited in Apple Aperture 3.5.1 and all final figures prepared in Adobe Photoshop Elements 12.0. Drawings and measurements were made using a camera lucida attachment on a Wild M8 stereo-zoom or Nikon Labophot-2 compound microscope. Morphometric analyses were made using Statview 4.5 on an Apple G4 Powerbook laptop in Classic mode. Cited material is deposited in the National Museum Wales, Cardiff (NMW).

7.3.3 *Molecular identification*

Using the HotSHot protocol, genomic DNA was individually extracted from 8 branchiobdellidans (4 of each species) in 99 µl TE buffer including 5 µl of proteinase K and 0.45% Tween 20 incubated at 56ºC for 4 h and neutralised at 95°C for 10 min (Truett et al. 2000). Universal mitochondrial cytochrome *c* oxidase I (COI) primers modified from Folmer et al. (1994), forward: 5' GGT CAA CAA ATC ATA AAG AYA TYG G 3', and reverse: 5' TAA ACT TCA GGG TGA CCA AAR AAY CA 3' or 5'-AAGAGCGACGGGCGATGTGT-3' (Harper et al. 2005) were used to amplify a ~700bp fragment. Higher quality sequences were obtained using the latter reverse primer. PCR consisted of 1 µl DNA template, 1.5 µl buffer, 1.5 µl magnesium chloride (25 mM), 0.3 µl ddNTP mix (10 mM), 1 µl of each primer (10 µM), 0.2 µl FermantasTaq DNA polymerase (5 U/µl), and water to a total volume of 15 µl. A negative control (with no DNA template) was included with each reaction. PCR conditions were as follows: initial 180s denaturation at 95°C; 35 cycles of 30s at 94°C, followed by 40s at 48°C and 70s at 72°C; final 12 min elongation at 72°C. PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase and sequenced using the same primers at Cardiff University Molecular Biology Support Unit. Forward and reverse amplicons were aligned and edited using Sequencher^{TM} version 4.7 and subsequently compared against all branchiobdellidan sequences available on Genbank.

7.3.4 *Statistical analysis*

Separate negative binomial generalized linear mixed models (GLMMs) were run to investigate factors influencing the infection intensity of each branchiobdellidan species on signal crayfish. Crayfish moult stage (inter-moult, pre and post-moult or in moult), size (carapace length, mm) sex/life stage (juvenile, male or female) and an interaction between the latter 2 variables, were

included in both models. Additionally, chelae number (0, 1 or 2) was included as a random effect in these GLMMs. Models were refined by stepwise deletions of non-significant terms from the starting model using Analysis of Variance (Crawley 2007). Model fit was assessed through visual examination of Pearson's residual plots, as according to Thomas et al. (2013). All data analyses were conducted using the glmmADMB package in R statistical software version 3.02.

7.4 Results

7.4.1 *Field survey*

Of the 17 sites surveyed for crayfish in Wales during July-October 2012, invasive signal crayfish (*Pacifastacus leniusculus*) were found at 9 and native white clawed crayfish (*Austropotamobius pallipes*) at 2 locations (Table 7.1; data added to CrayBase, see Chapter 3). Branchiobdellidans were only found at 1 site in the River Gavenny (Ordnance Survey Grid Reference: SO308164), Abergavenny, Wales, on 18.5% of signal crayfish (5 out of 27) screened in 2012. A further 73 crayfish were collected in the 5 surveys conducted at the River Gavenny between April and June 2013. Two species of branchiobdellidans were found, subsequently identified through morphological and molecular analysis as *Xironogiton victoriensis* and *Cambarincola* aff. *okadai*. The prevalence of *X. victoriensis* and *C.* aff. *okadai* across these surveys were 75.34% and 71.23% respectively. Mean infection intensities were 52.93 (range: 1-272) and 3.88 (range: 1-18) for *X. victoriensis* and *C.* aff. *okadai* respectively.

Table 7.1. Total number, number of crayfish per hour (catch-per-unit-effort, CPUE) and species of crayfish (either invasive *Pacifastacus leniusculus* or native *Austropotamobius pallipes*) found at each of the sites (approximate National Grid References, NGRs, included) manually surveyed during July-Oct 2012.

Site name	NGR	No. crayfish	CPUE	Species
Nant Glandulas	ST194839	1	0.25	*P. leniusculus*
Bachowey 1	SO158464	2	0.25	*P. leniusculus*
Bachowey 2	SO168457	37	4.44	*P. leniusculus*
Sirhowey 1	ST178961	19	3.38	*P. leniusculus*
Gavenny	SO308164	27	3.95	*P. leniusculus*
Mochdre	SO086904	32	5.13	*P. leniusculus*
Lugg at Humber	SO522523	23	3.45	*P. leniusculus*
Back Brook	SO302569	11	1.78	*A. pallipes*
Dulas Brook	SO353321	1	0.2	*A. pallipes*
Gurrey Fach	SN622252	0	N/A	N/A
Sirhowey 2	ST184995	0	N/A	N/A
Dowlais Brook	ST309927	0	N/A	N/A
Knobley Brook	SO279607	0	N/A	N/A
Curl Brook	SO333570	0	N/A	N/A
Dore at Peterchurch	SO344385	0	N/A	N/A

Among infested hosts, co-infection with *X. victoriensis* and *C.* aff. *okadai* was common, with 75.41% of signal crayfish harbouring both species, but there was evidence of niche segregation with *X. victoriensis* found mainly on the chelae and *C.* aff. *okadai* mostly on the carapace (Fig. 7.1). Total branchiobdellidan burden was positively correlated with crayfish size (carapace length, mm) for *X. victoriensis* (GLMM, $LRT_{1, 65} = 100.13$, $P < 0.0001$) and *C.* aff. *okadai* ($LRT_{1,63} = 29.70$, $P < 0.0001$) (Fig. 7.2). Branchiobdellidan burden was also influenced by moult stage for *X. victoriensis* (GLMM, $LRT_{3, 65} = 10.94$, $P = 0.01$) and *C.* aff. *okadai* ($LRT_{3, 63} = 15.40$, $P = 0.002$) (Fig. 7.3). For *X. victoriensis* worm burdens were highest on pre or post-moult crayfish and lowest on crayfish in the process of moulting whereas for *C.* aff. *okadai* they were highest on inter-moult crayfish and lowest on pre or post-moult crayfish (although mean numbers of *C.* aff. *okadai* on pre and post-moult and in moult crayfish were similar) (Fig. 7.3). For *C.* aff. *okadai* branchiobdellidan burden was also significantly affected by crayfish sex/life stage (GLMM, $LRT_{1,63} = 6.45$, $P = 0.04$) with juveniles having the fewest worms and males and females having similar higher numbers. No branchiobdellidans were found in the branchial chambers of dissected crayfish.

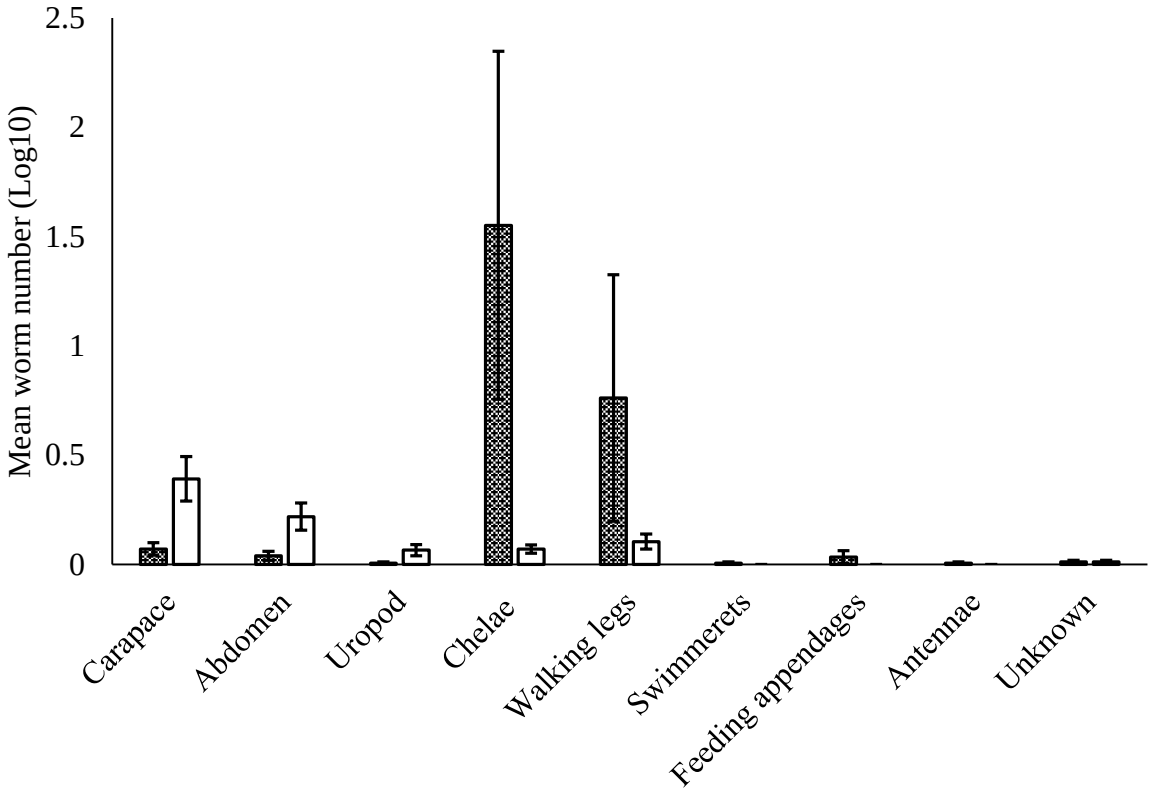

Fig. 7.1. Mean ± SE (Log10) number of branchiodellidans of *Xironogiton victoriensis* (white bars) and *Cambarincola* aff. *okadai* (hatched bars) on different regions of the crayfish host, *Pacifastacus leniusculus*.

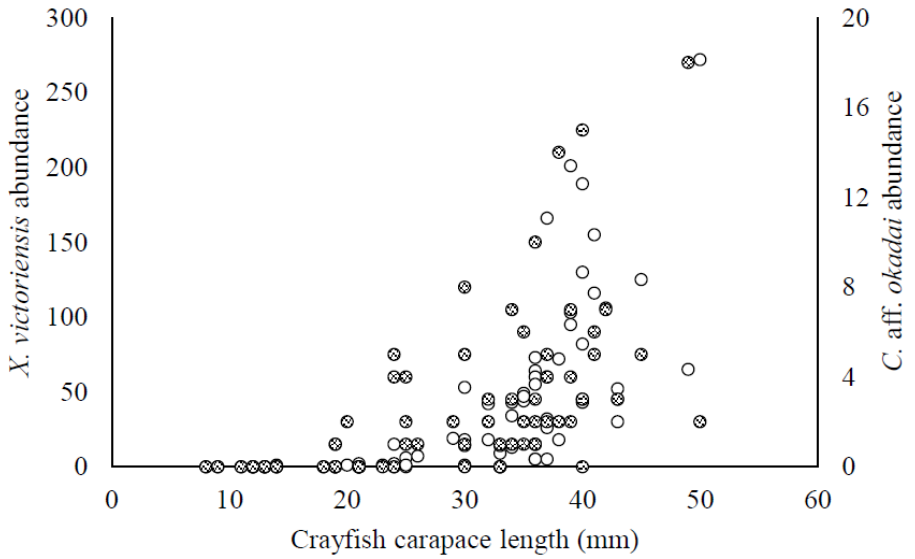

Fig. 7.2. Correlation between crayfish size (carapace length, mm) and abundance of *Xironogiton victoriensis* (white circles) and *Cambarincola* aff. *okadai* (hatched circles).

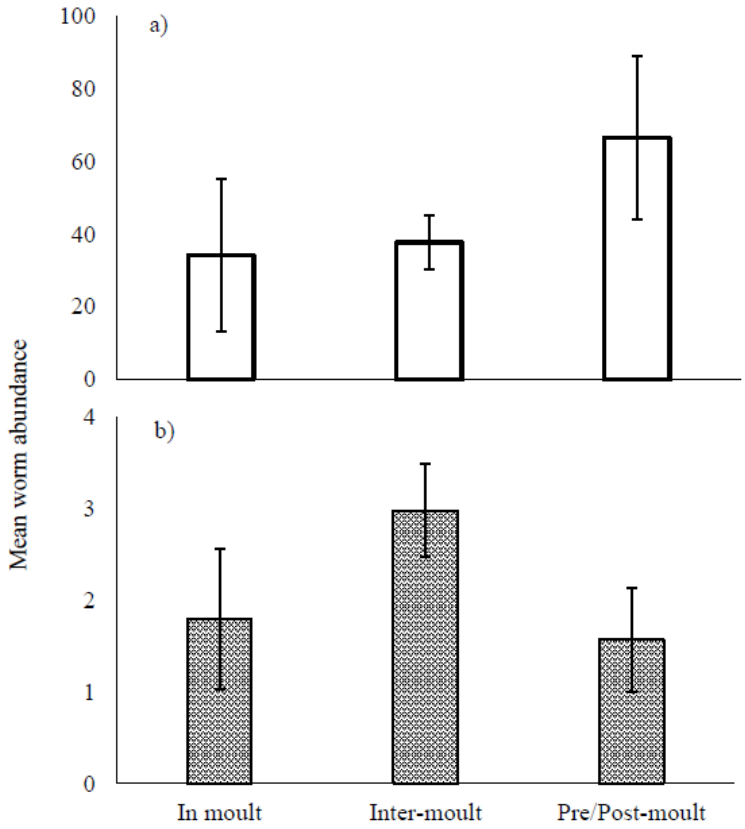

Fig. 7.3 Mean (±SE) abundance of a) *Xironogiton victoriensis* and, b) *Cambarincola* aff. *okadai* on in moult, inter-moult and pre or post-moult crayfish.

7.4.2 *Morphological identification*

Xironogiton victoriensis Gelder and Hall, 1990

(Fig. 7.4 and 7.5)

Material examined: Gavenny River, Abergavenny, southeast Wales, on chelae and walking legs of signal crayfish, *Pacifastacus leniusculus*, coll. J. James: 6 specimens, 23.04.14 (NMW.Z.2014.012.012-16); 4 specimens, 17.04.13 (NMW.Z.2014.012.017); 9 specimens, 20.05.14 (NMW.Z.2014.012.018); 13 specimens, 21.05.14 (NMW.Z.2014.012.019); jaws, slide preparation (NMW.Z.2014.012.020).

Description: Colour in life (Figs. 7.4A-C) generally transparent to white, with green-brown gut and white reproductive organs discernable to varying extent; brown jaws visible through cuticle at peristomium-head junction. Fixed or preserved specimens opaque, white.

Exemplar live animal (Fig. 7.4A) varying from short, anteriorly sub-cylindrical and posteriorly broad, flask-shape (total length 2.6 mm, maximum width 1.2 mm), to elongated pyriform (4.9 mm, 0.8 mm); segments 5-8 dorsally convex, ventrally flattened to concave with narrow skirt-like lateral margins.

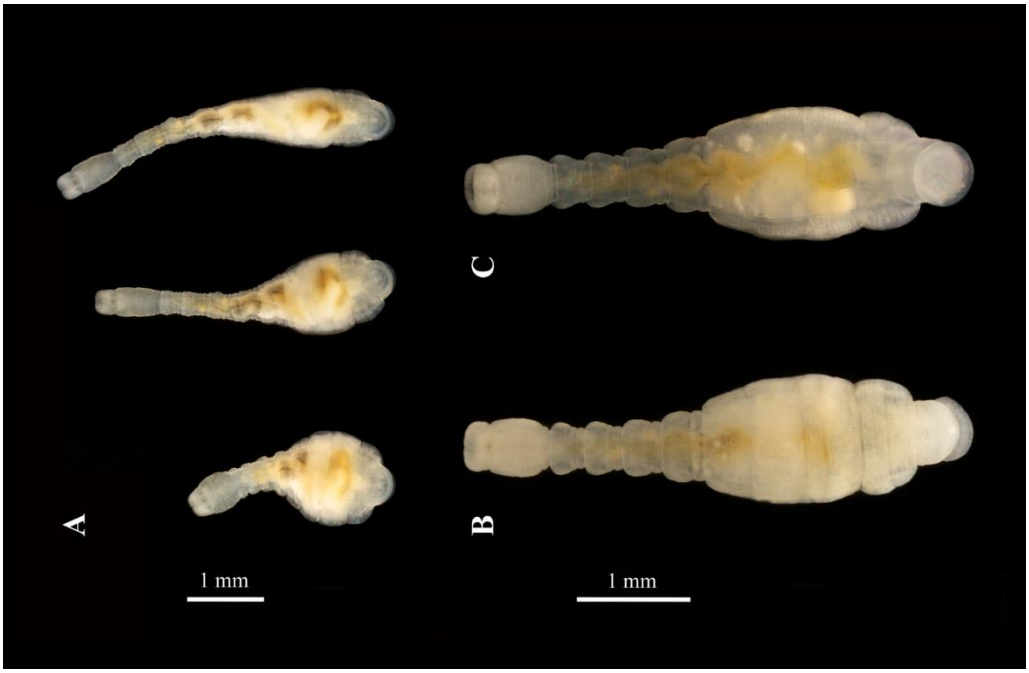

Fig. 7.4. *Xironogiton victoriensis* (A) live animal, 3 dorsal views (NMW.Z.2014.012.013); (B, C) $MgCl_2$ narcotized animal, dorsal and ventral views (NMW.Z.2014.012.012).

Animals narcotized by exposure to $MgCl_2$ pyriform in shape (Figs. 7.4B, C); when subsequently formalin fixed, short to moderately long (total length 1.6-4.3 mm, mean 3.2 mm, $n = 4$), pyriform (maximum width 0.56-1.19 mm, mean 0.92 mm, $n = 3$); length:width ratio 3.2-5.5, mean 4.3, $n = 3$; head region:total length ratio 0.17-0.20, mean 0.185, $n = 4$. Direct ethanol

preserved specimens (Fig. 7.5A) short (1.5-2.7 mm, mean 2.1 mm, *n* = 28), broad flask shaped (width 0.56-1.44 mm, mean 1.05 mm, *n* = 27); length: width ratio 1.6-3.0, mean 2.0, *n* = 27; head region: total length ratio 0.18-0.25, mean 0.214, *n* = 28.

Head region comprising peristomium and head, former delimited by distinct anterior constriction. Peristomium short, about a third as long as the head; upper lip usually bilobed, lower lip straight to broadly emarginate, surfaces adorned with small groups of stiff cilia; no tentacles. Dorsal jaws visible, with alternating dark brown-light brown longitudinal banding. Oral papillae difficult to discern, digitiform, 16, arising around mouth and anterior to the jaws (Figs. 7.5B, C). Dorsal and ventral jaws similar, rounded rectangular plates with posteriorly directed teeth. Dental formulae exhibiting only slight variation: 5/4, 5/5 (Figs. 7.5D, E) and 4/5 (Figs. 7.5B, C) observed. Teeth unequal, middle tooth shortest, 1 lateral often a little more robust and curved.

Anterior nephridiopores not seen. Spermathecal pore mid-ventrally on segment 5, short clavate spermatheca visible through cuticle. Male genital pore on segment 6 more conspicuous, with slightly raised margins (Fig. 7.5A). Anus conspicuous in live animals, opening on segment 10, dorsal to the sucker (Figs. 7.4A, B). Posterior sucker a circular disc.

Remarks: The shape of the species varies greatly in life and with respect to the fixation or preservation procedure. Our morphometric analyses show that, while the relationship of the head region to overall length was similar (mean values of 18.8 and 21.4%), total length to width ratios differed markedly (mean values 4.3 vs 2.0) between $MgCl_2$ narcotized formalin fixed and direct ethanol (100%) preserved specimens.

The characteristics of the Welsh material correspond well with the original description from British Columbia, Canada (Gelder and Hall 1990) and subsequent records from Europe. The light-dark brown banding of the teeth was consistent with the appearance of the jaws photographed by Oberkofler et al.'s (2002) Fig. 8 and Geasa's (2014) Fig. 4N. The first European record of *X. victoriensis* (as *Xironogiton instabilis* Moore, 1893; see Gelder et al. 2012) was from Sweden (Franzén 1962). Since then, the species has been found further south in Spain (Gelder 1999; Oscoz et al. 2010), Italy (Quaglio et al. 2001; Oberkofler et al. 2002; Gelder 2004), Hungary (Kovács and Juhász 2007), Austria (Nesemann and Neubert 1999 cited in Subchev 2008), France (Gelder et al. 2012) and potentially Finland (Kirjavainen and Westman 1999). Klobucar et al. (2006) stated that the species "is almost certainly to be found wherever the [signal] crayfish has been introduced." In terms of the current study *X. victoriensis* was only found in 1 signal crayfish population but we do not have details on the timing or

county of origin of most British signal crayfish populations, so are unable to assess the relevance of our finding.

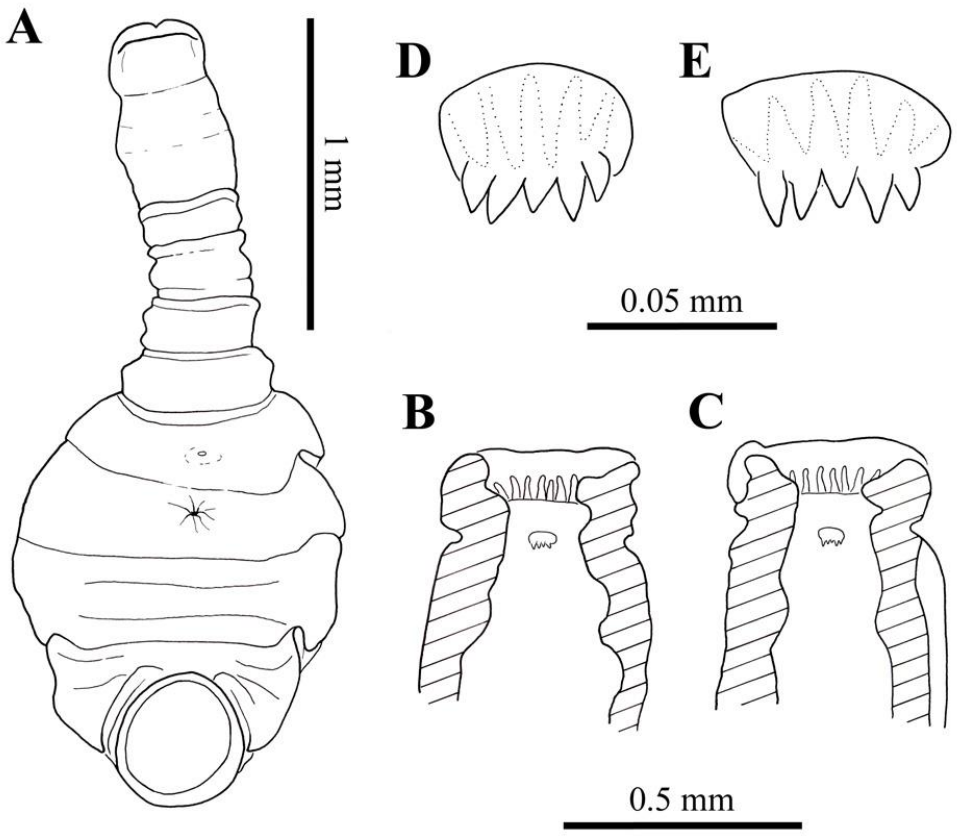

Fig. 7.5. *Xironogiton victoriensis* (A) formalin fixed specimen, ventral view (NMW.Z.2014.012.013); (B, C) anterior pharyngeal region, frontal plane section, showing dorsal and ventral jaws and most oral papillae (NMW.Z.2014.012.012); (D, E) dorsal and ventral jaws, viewed from pharynx (NMW.Z.2014.012.013).

Cambarincola aff. *okadai* Yamaguchi, 1933

(Figs. 7.6 and 7.7)

Material examined: Gavenny River, Abergavenny, southeast Wales, at the dorsal cephalothorax and abdomen of signal crayfish, *Pacifastacus leniusculus*, coll. J. James: 4 specimens, 17.04.13 (NMW.Z.2014.012.001-2); 10 specimens, 23.04.14 (NMW.Z.2014.012.003-8); 14 specimens, 20.05.14 (NMW.Z.2014.012.009); 3 cocoons, 23.04.14 (NMW.Z.2014.012.010); jaws, slide preparation (NMW.Z.2014.012.011).

Description: Colour in life (Figs. 7.6C-E) yellow-white, with paler white-transparent head and posterior sucker regions; dark gut contents visible for most of body length. Brown jaws indistinctly visible through cuticle at peristomium-head junction (Fig. 7.6C); more clearly seen through mouth opening (Fig. 7.6B). Fixed or preserved specimens opaque, white (Fig. 7.6A).

Exemplar live animal sub-cylindrical (Fig. 7.6C), varying from short and stout (total length 7.5 mm, maximum body segment width 1.07 mm) to long and narrow (13.2 mm, 0.80 mm). Maximum observed length of other animals in petri dish, 17 mm.

Narcotized animals sub-cylindrical (Figs. 7.6D, E); when subsequently formalin fixed, moderately long (4.1-8.2 mm, mean 7.1 mm, *n* = 8) and wide (0.61-1.20 mm, mean 0.96 mm, *n* = 5); length: width ratio 6.6-7.7, mean 7.0, *n* = 5; head region: total length ratio 0.18-0.22, mean 0.194, *n* = 8. Ethanol preserved specimens somewhat dorso-ventrally flattened (Fig. 7.6A, 7.7A), short to moderately long (4.6-8.3 mm; mean 6.0 mm, *n* = 20) and stout (width 1.06-1.67 mm, mean, 1.35 mm, *n* = 19); length: width ratio 3.9-5.2, mean 4.4, *n* = 19; head region: total length ratio 0.18-0.24, mean 0.215, *n* = 20.

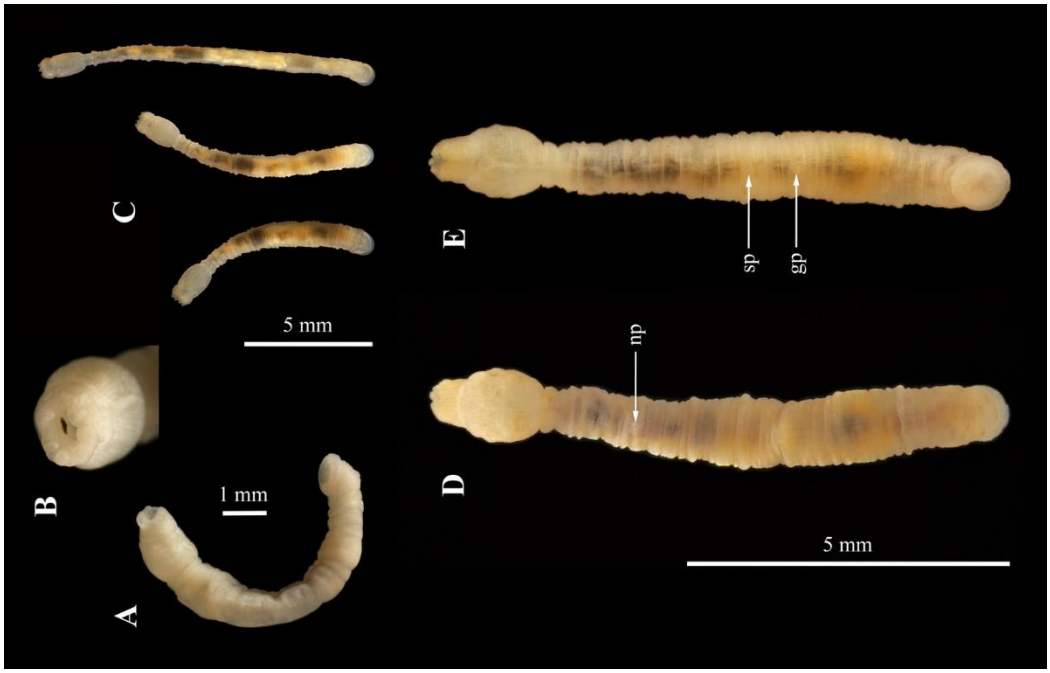

Fig. 7.6. *Cambarincola* aff. *okadai* (A) 100% ethanol preserved specimen, lateral view; (B) same, anterior, oblique ventral view (A, B: NMW.Z.2014.012.001); (C) live animal, 3 dorsal views (NMW.Z.2014.012.008); (D, E) $MgCl_2$ narcotized animal, dorsal and ventral views (NMW.Z.2014.004). gp, male genital pore; np, nephridial pore; sp, spermathecal pore.

Head region comprising peristomium and head, former delimited by distinct anterior constriction. Peristomium short, about 30% of the head length. Peristomial shape very variable in live animals, from narrow cylindrical to anteriorly wide funnel-like; in alcohol preserved specimens, often more constricted and anteriorly tapering, somewhat conical. Upper lip bearing 2 pairs of short tentacles, medial gap between pairs larger than separation of tentacles in each pair (Figs. 7.6B, C), Shape of tentacles variable relative to degree of contraction or expansion of peristomium, ranging from blunt rounded lobes (Figs. 7.6A, B, D, E, 7.7A) to more pointed conical (Fig. 7.6C). When peristomium expanded and anteriorly flared in live animals, or when

oral papillae are extruded (Fig. 7B), the tentacles become reduced and almost disappear, with only short blunt tips remaining. Ventral lip bilobed with 2 broadly rounded lobes (Figs. 7.6B, E; 7.7A, B, D). Weakly defined lobes laterally (2 or 3, usually 3), and even more indistinct 'lobes' between each tentacle pair (2 or 3) and between tentacles in each pair (1 or 2). Head cylindrical to barrel-shaped, wider than anterior segments in live animals.

Oral papillae difficult to discern unless protruded (Fig. 7.7B) or head region dissected (Figs. 7.7C, D). Papillae triangular, 16, arising around mouth and anterior to the jaws. Jaws subequal, broad (up to ca. 220 µm across), triangular, each with a single large bluntly triangular tooth and 2 pairs of small secondary teeth laterally, on exposed oral (dorsal) surfaces; dentition 5/5. Secondary teeth sharper, more projecting and readily observed on pale brown 'younger' jaws; 'older' jaws very dark brown, robust with low bluntly rounded secondary teeth (Figs. 7.7E, F). Secondary teeth can often only be confirmed through examination using a compound microscope; jaws appearing unidentate when viewed using a stereo-zoom dissecting microscope.

Body segments primarily triannulate, though further lesser annulations also occur. Low thickened ring additionally present on each segment in live animals (Figs. 6C-E), most obvious on segments 4-8; pronounced raised dorsal ridges absent. Thickened rings indistinct on fixed and preserved specimens.

A single mid-dorsal nephridiopore occurs on segment 3 (Fig. 7.6D). The nephridiopore sometimes inconspicuous, but often revealed through the use of Methyl Green or Shirlastain A staining. Segment 4 with a long strap-like nephridium, distal part lying transversely above the gut at posterior of segment, proximal part passing ventrally and anteriorly into segment 3. Spermathecal pore mid-ventrally on segment 5. Male genital pore on segment 6 more conspicuous, often with clearly protruding margins (Figs. 7.6E, 7A). Posterior sucker a large circular and concave disc.

Spermatheca variable, ranging from having a short duct leading to a longer distally rounded cylindrical structure (Fig. 7.7G) to a long narrow duct and a wide globular distal bulb. Male genitalia with long tubular prostate gland positioned above the glandular atrium, distal region of latter varying from a Y-shape, curving round the bursa (Figs. 7.7H, I), to a more triangular, less indented structure. Methyl Green staining deepest in glandular atrium, moderate in bursa and weak in prostate.

Stalked cocoons (from crayfish cuticle) transparent with variable amount of red-brown stellate ornamentation, each containing a single larva. Larvae active, moving within cocoon, hatching from the top when sufficiently developed.

Remarks: The total length to width relationship of this large species varies greatly in life and with respect to the fixation or preservation procedure. While the head region to overall length relationship was similar (mean values of 19.4 and 21.5%), length to width ratios were quite distinct (mean values 7.0 vs 4.4) between $MgCl_2$ narcotized formalin fixed and direct ethanol (100%) preserved specimens.

The upper peristomial lip of the Welsh species bore 4 long dorsal tentacles, a character shared with 4 species from North America: *C. okadai* Yamaguchi 1933 (a North American introduction to Japan), *C. macrocephalus* Goodnight 1943 (Wyoming), *C. fallax* Hoffman, 1963 (Virginia), and *C. holti* Hoffman, 1963 (Kentucky). Holt (1974) redescribed *Triannulata montana* Goodnight 1940 (Washington) and transferred it to *Cambarincola*, while Gelder and Ohtaka (2000) later synonymized it with *C. okadai*. Several other species – *C. philadelphicus* (Leidy, 1851: Pennsylvania), *C. chirocephalus* Ellis, 1919 (Missouri), *C. ingens* Hoffman, 1963 (Virginia), and *C. gracilis* Robinson, 1954 (see Holt 1981) – have 4 small lobes on the dorsal lip but, according to Hoffman (1963), this condition "in no way approximates the conspicuous tentaculation of *fallax* and some other species."

Other consistent features of the Welsh material were a lower lip bearing 2 large broadly rounded lobes, and large triangular jaws armed with a single strong distal tooth and 2 pairs of small dorsolateral secondary teeth. However, the specimens exhibited intraspecific variations in the morphology of the spermatheca and male reproductive apparatus that encompassed some of the interspecific differences used in distinguishing the 'tentaculate' species in the comprehensive (though sometimes ambiguous) key to 48 species of *Cambarincola* presented by Holt and Opell (1993).

Hence a distally bilobed to slightly emarginate triangular glandular atrium, together with a prostate of similar or slightly shorter length, could be considered common to *C. okadai*, *C. macrocephalus*, *C. fallax*, *C. holti*, *C. philadelphicus* and *C. chirocephalus*. *Cambarincola ingens* is distinguished by having an additional long sinuous or coiled distal extension to the prostate (Hoffman's 1963 Fig. 34; Holt and Opell's 1993 Fig. 95). All these species have spermathecal morphologies encompassed in the variation observed in the Welsh specimens, except *C. holti* that has an additional tubular and highly glandular distal extension (Hoffman's 1963 Fig. 60; Holt and Opell's 1993 Fig. 89). *Cambarincola gracilis* differs from all the above in having a conspicuously shorter prostate gland (see Holt's 1981 Fig. 2C).

Four species (*C. philadelphicus*, *C. fallax*, C. *chirocephalus* and *C. gracilis*) are reported to possess "prominent dorsal ridges" (Holt and Opell 1993) in preserved specimens. These dorsal ridges may actually encircle the segments (e.g., in *C. gracilis*; see Gelder et al.'s 2012

Fig. 1). By contrast, the low thickened segmental rings, observed in the live Welsh animals, were absent or scarcely visible in non-narcotized ethanol preserved specimens.

The morphology of the Welsh species most closely resembles that of the remaining 2 species, *C. okadai* and *C. macrocephalus*, though the latter could be separated from both by having a head region "approaching $^{1}/_{3}$ entire body in size" (Holt 1981) and this feature was used as an early separator in Holt and Opell's key (1993). There are nevertheless similarities in the body annulation pattern, peristomial tentacles and lobes, and jaws. Considering first the body annulations; Goodnight (1940) originally created a new genus *Triannulata*, for *T. magna* and *T. montana* (latter currently a junior synonym of *C. okadai*), in acknowledgement of the secondary annulations evident on each segment. Gelder and Ohtaka (2000) confirmed the presence of triannulate segments in their redescription of *C. okadai*. Goodnight (1943) in his original description of *C. macrocephalus* referred to the major annulations of the body segments as "not elevated over minor annulations" and, in his redescription of the holotype, Hoffman (1963) noted that some of the anterior segments "notably from III to VI, are quite distinctly tripartite." However, Holt (1981) dismissed Hoffman's observation as a misinterpretation of a slide preparation.

Regarding the peristomial structures, the infrequently reported *C. macrocephalus* was recorded (Hoffman 1963) as having "4 distinct slender submarginal tentacles" dorsally and as being "broadly bilobed" ventrally. This is very similar to the account of the peristomium in the original description of *C. okadai*; the dorsal part "having 4 distinct digitiform appendages, while the ventral part is thick and slightly bilobed" (Yamaguchi 1933). Its junior synonym *T. montana* was described as having its peristomium divided in to "12 lobes (4 dorsal, 4 ventral, and 4 lateral) which may be extended into tentacular appendages, dorsal longer than ventral or lateral" (Goodnight 1940). This arrangement was confirmed by Holt (1974), who distinguished the tentacles of *C. montanus* n. comb. from the lobes, "4 dorsal tentacles, 2 lateral lobes either side and 4 ventral lobes." Holt also remarked that variations in the length of the tentacles were of no consequence, and that oral papillae were not detectable. In a later account, Holt (1981) referred to the lateral and ventral lobes as "prominent". Gelder and Ohtaka (2000) redescribed *C. okadai* and reported the peristomium as having a "dorsal lip with 4 distinct lobes (l) (or tentacles), 2 pairs of lateral lobes, and a ventral lip (v) consisting of a pair of short lobes laterally and a central portion with a slight median incision." In the Welsh material, we found a ventral lip of 2 broadly rounded lobes and above these, either side, up to 3 smaller, weakly defined, lateral lobes. Should the most inferior of these lateral lobes be considered 'ventral' then the Welsh

specimens would be in complete agreement with Gelder and Ohtaka (2000) Fig. 1B (reproduced in Gelder et al.'s 2012 Fig. 6).

Literature accounts of jaw dentition in *Cambarincola* are often quite variable and this may reflect natural variation, differences between young and old jaws, inadequate microscopical examination, differences between populations, or a compounding of different species. Goodnight (1943) discovered *C. macrocephalus* on the Pilose Crayfish, *Pacifastacus gambelii* (Girard, 1852); mature worms were up to 4 mm long. The jaws were described as "large triangular blocks of chitin terminating in sharp tooth. Without lateral teeth but margin uneven. Width of jaws at base 400 μm." Hoffman (1963) re-examined the holotype and, with some reservation, some material from a new locality in Idaho. Preserved worms were up to 4.8 mm (0.8 mm wide) with a head region up to 1.1 mm wide, and the jaws large and robust with 3/3 dentition. Holt (1981) emended Hoffman's description and cited preserved specimens up to 5.6 mm long and 1.4 mm wide. The jaws were interpreted as having a single tooth, "though there appear to be lateral teeth on the jaws of younger animals" and the latter were illustrated (Holt 1981: Fig. 3C) with a 3/3 dentition. Recently, Geasa (2014) recorded *C. macrocephalus* among the branchiobdellidans found on signal crayfish (*Pacifastacus leniusculus*) from 3 out of 7 locations in British Columbia, western Canada. Length was recorded as up to 7 mm, and the jaws described as having 1/1 dentition.

In the original description of *C. okadai*, Yamaguchi (1933) described worms up to 7 mm long and 0.8 mm wide, from signal crayfish introduced to Japan. The jaws were triangular with a large conical tooth and 2 small denticles either side. Drawings of the jaws by Yamaguchi (1933: Fig. 2A, B) and in the redescription by Gelder and Ohtaka's (2000) Fig. 1C, D; (see also Gelder et al.'s 2012 Fig. 7) are morphologically identical to those found in young Welsh specimens. Gelder and Ohtaka (2000) noted a discrepancy regarding total length in the type material and found it to be up to 4.7 mm; jaws were 95-130 μm wide. Despite this, Gelder et al.'s (2012) Fig. 2 presented a photograph of a 7 mm long adult from France. Goodnight (1940) described the triangular jaws of *T. montana* as measuring 250 μm across in a worm 5 mm long (1.25 mm wide); dentition 7/5, each with a longer median tooth and smaller lateral ones. Holt (1974) subsequently reported *C. montanus* n. comb. as being up to 6.3 mm long and 1.0 mm wide; dentition usually 1/1, but 5/5 in younger, though large, specimens from the type locality. In a later paper, Holt (1981) inexplicably gave the dentition as 1/2, and 5/5 in immature forms. However, Gelder and Hall's (1990) Fig.D jaws of sexually mature specimens from British Columbia with 5/5 dentition. An SEM image of the peristomial tentacles and lobes in *C.*

montanus from British Columbia (Geasa's 2014 Fig. 6C) is strikingly similar to their appearance in Welsh *Cambarincola* material.

Consideration of all the morphological information shows that the Welsh specimens have the closest affinity with *C. okadai*, but are noticeably larger (at least 8.3 mm long, preserved; live animals extending to at least 17 mm), with larger jaws (195-220 µm wide). This jaw size approaches that indicated for *T. montana* and raises questions about its synonymy with *C. okadai*. Indeed, *C. okadai* as currently understood could conceivably encompass an even larger number of cryptic species. Misidentification of branchiobdellidans is common. For example, Williams et al. (2013) found a 49% error in Genbank sequences, and their molecular analyses indicated that *C. philadelphicus sensu lato* comprised at least 4 cryptic (and unrelated) taxa. A similar situation may well exist in a *C. okadai–C. montanus* group and this can only be resolved by a combined morphological and molecular study of material from western USA and Canada, and Japan.

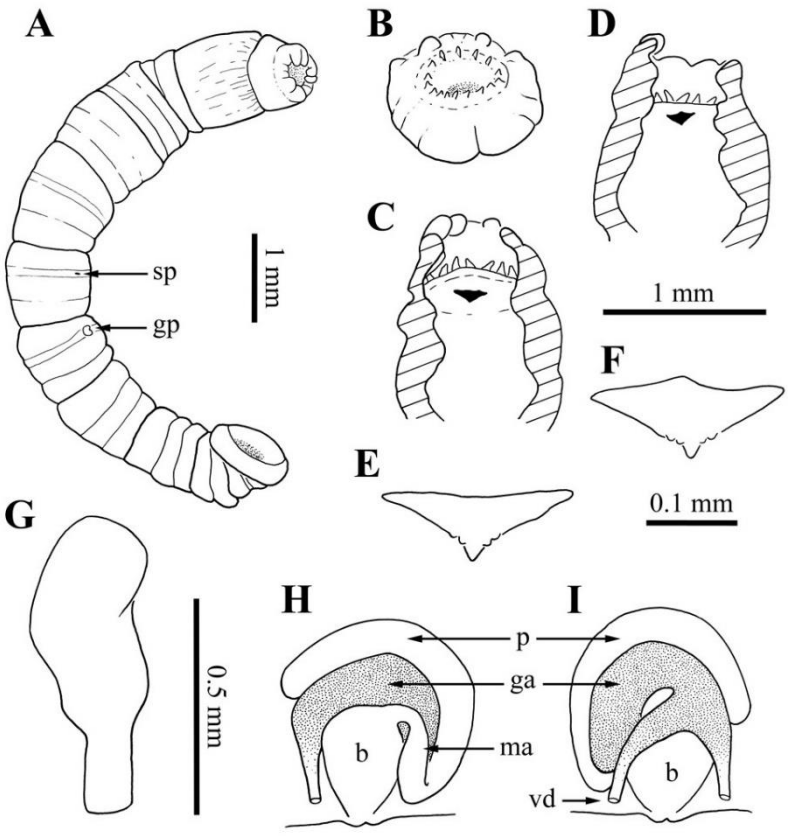

Fig. 7.7. *Cambarincola* aff. *okadai* (A) 100% ethanol preserved specimen, lateral view (NMW.Z.2014.012.001); (B) formalin fixed specimen, anterior with 16 oral papillae exposed, oblique ventral view (NMW.Z.2014.012.003); (C, D) anterior pharyngeal region, frontal plane section, showing dorsal and ventral jaws and most oral papillae; (E, F) dorsal and ventral jaws, viewed from pharynx; (G) spermatheca; (H, I) male genitalia, left and right lateral views (C-I: NMW.Z.2014.001). b, bursa; ga, glandular atrium; gp, male genital pore; ma, muscular atrium; p, prostate; sp, spermathecal pore; vd, vas deferens.

7.4.3 *Genetic analysis*

The mitochondrial COI partial sequence (653 bp) obtained from the *X. victoriensis* specimens (Genbank Accession Number KT025254) was 99% similar to *X. victoriensis*, Genbank Accession Number: JQ821631.1, differing at base pairs 265 (G: A), 340 (C: T) and 581 (T: C). A 99% sequence match for *X. victoriensis* specimens was also obtained for *Sathrodrilus attenuatus* (AF310719.1), differing at base pairs 300 (G: A), 450 (G: A) and 523 (C: T), which Williams et al. (2013) indicated was a misidentified *X. victoriensis* specimen. The best quality sequence obtained for the *Cambarincola* aff. *okadai* species was a 598 bp fragment (Genbank Accession KT025253) with 1 nucleotide ambiguity (at base pair 134) which was a 91% match to *Cambarincola montanus* (synonymized with *C. okadai* by Gelder and Ohtaka 2000), Genbank Accession Number: AF310711.1.

7.5 Discussion

We provide the first record of non-native branchiobdellidans on invasive crayfish in the British Isles. Both identified branchiobdellidan species, *Xironogiton victoriensis* and *Cambarincola* aff. *okadai* had a relatively high prevalence of 75.34% and 71.23% respectively in the infected signal crayfish (*Pacifastacus lenisuculus*) population. Further studies are required to determine the potential community level consequences of this symbiotic relationship.

X. victoriensis is native to Canada (Gelder and Hall 1990) and within Europe was first reported in Sweden (Franzén 1962) and subsequently Spain (Gelder 1999; Oscoz et al. 2010; Vedia et al. 2014), Italy (Quaglio et al. 2001; Oberkofler et al. 2002), Hungary (Kovács and Juhász 2007), Austria (Nesemann and Neubert 1999 cited in Subchev 2008), France (Laurent 2007; Subchev 2008; Gelder et al. 2012) and potentially Finland (Kirjavainen and Westman 1999). It should be noted that Nesemann and Neubert (1999; cited in Subchev 2008) and Kovács and Juhász (2007) all identified their *Xirongiton* specimens as *X. instabilis* but it is almost certain that these are samples of *X. victoriensis* that were misidentified (Subchev 2008). In Europe, *X. victoriensis* are mostly found on North American signal crayfish (Franzén 1962; Kirjavainen and Westman 1999; Gelder 1999; Quaglio et al. 2001; Oberkofler et al. 2002; Kovács and Juhász 2007; Laurent 2007; Subchev 2008; Oscoz et al. 2010; Gelder et al. 2012), which they infest in their native range (Gelder and Hall 1990). In Spain, *X. victoriensis* have however, recently been recovered from Louisiana red swamp crayfish (*Procambarus clarkii*) with which they do not naturally co-exist (Vedia et al. 2014). Because of the generalist host range of *X. victoriensis* all invasive crayfish species in the UK should be considered as a potential host, and thus transmission pathway, for this symbiont.

Cambarincola okadai, the closest identified relative of *C.* aff. *okadai*, is native to North America (Yamaguchi 1933) and within Europe has only been recorded on signal crayfish from France (Gelder et al. 2012). As the specimens of *C. okadai* from France were identified only through morphological techniques (Gelder et al. 2012) it is possible that they are actually the same species as the *C.* aff. *okadai* described in the current paper, genetic analysis of French *C. okadai* would be needed to test this. Additionally, the specimens of *C.* aff. *okadai* described in the current study exhibit some morphological similarity with *C. macrocephalus* (see Goodnight 1943). However, as no genetic sequence is currently available for the latter, we are unable to determine whether this is the first record of a new species of branchiobdellidan or range extension of an existing one. Nevertheless, the current study is the first report of *C.* aff. *okadai*, as well as *X. victoriensis*, in the British Isles, representing an increase in the global spread of branchiobdellidans.

In the current study branchiobdellidans were only present at 1 of the 9 sites positive for invasive signal crayfish. Further, there were no reports of branchiobdellidans in any of the 7,161 signal crayfish records analysed from the UK national crayfish database, CrayBase (Chapter 3). This includes surveys conducted in the same river catchment, the Usk, as the branchiobdellidan infested population in the River Gavenny (FM Slater personal communication). The absence of branchiobdellidans in nearby signal crayfish populations suggests that their introduction into the Gavenny occurred fairly recently. Furthermore, records of native white clawed crayfish (*Austropotamobius pallipes*) in the Gavenny from 2000 implies that the introduction of signal crayfish into this river may also have occurred as recently as the last decade or 2 since natives are typically displaced by signal crayfish.

The consequences of *X. victoriensis* and *C.* aff. *okadai* introduction for British crayfish are unknown as the relationship between crayfish and branchiobdellidans varies from parasitism (e.g. Rosewarne et al. 2012) to commensalism (e.g. Keller 1992) to mutualism (e.g. Brown et al. 2002, 2012; Lee et al. 2009) depending on the worm and crayfish species, environmental conditions (Lee et al. 2009) and branchiobdellidan density (Brown et al. 2012). Branchiobdellidan densities on British crayfish ranged from 1-272 for *X. victoriensis* and 1-18 for *C.* aff. *okadai* and were influenced by crayfish size, moult status and, for *C.* aff. *okadai*, sex. As observed in previous studies (e.g. Keller 1992; Brown and Creed 2004; Skeleton et al. 2014) the abundance of both branchiobdellidans, increased with crayfish size. Larger crayfish have a greater surface area available for branchiobdellidan colonization and moult less frequently which is important as *X. victoriensis* and *C.* aff, *okadai* reside on the exoskeleton of the crayfish (Koepp 1975). Additionally, larger crayfish may also exhibit reduced grooming responses to

branchiobdellidans than smaller individuals, potentially contributing to the frequently observed relationship between crayfish size and branchiobdellidan abundance (Skelton et al. 2014). Age-specific variation in grooming has, however, only been observed in branchiobdellidans known to clean epibionts from crayfish gills (Skelton et al. 2014). Such ontogenetic changes in grooming behaviour are predicted to reflect age related shifts in the cost-benefits of the symbiosis to the crayfish host (Skelton et al. 2014). We did not, however, observe either *X. victoriensis* or *C.* aff. *okadai* in the branchial chambers of crayfish, and so there is no evidence that they are involved in a cleaning symbioses with the host. Indeed we found that moult phase had a significant effect on the abundance of both worm species. As we were, however, not able to distinguish between pre and post-moult crayfish we do not make any specific predictions regarding the relationship between crayfish moult stage and branchiobdellidan abundance. *C.* aff. *okadai* abundance was also influenced by crayfish sex/life stage, being lower on juvenile crayfish than either adult males or females. This may because of the smaller area available for colonization by *C.* aff. *okadai,* which are relatively large branchiobdellidans, on juvenile crayfish. We, however, found no evidence of a significant interaction between crayfish size and sex/life stage in explaining variation in *C.* aff. *okadai* abundance. Therefore increased moulting frequency of juveniles may be more important that size in determining *C.* aff. *okadai* abundance. .

We observed a high level of co-infection between *X. victoriensis* and *C.* aff. *okadai* therefore it is likely that crayfish migrating from the infected population will harbour both species. Interspecific competition may alter the effect of 1 or both branchiobdellidan species on the crayfish host by causing worms to switch their micro-habitat or feeding preference. We found evidence of *X. victoriensis* and *C.* aff. *okadai* micro-habitat niche segregation on crayfish hosts but, due to a paucity of uninfected crayfish, it was not possible to determine if this was the result of interspecific competition. We found no worms of either species in the branchial chamber of dissected infected crayfish although only a small number of crayfish were examined (n = 10).

Overall, the current study documents the presence of 2 novel symbiotic annelids in the British Isles, 1 of which may be a previously undescribed species. Future work involving comparative sequencing of mitochondrial CO-I from *C. okadai* and *C. macrocephalus* populations is needed to elucidate the identity of *C.* aff. *okadai*. This highlights the need to improve the current sequence data available for branchiobdellidans, something that has already been demonstrated by Williams et al. (2013). Experiments assessing the nature of the relationship between crayfish and *X. victoriensis* and *C.* aff. *okadai,* are needed to assess the

potential consequences of branchiobdellidan infection for both invasive signal and endangered native crayfish in Britain.

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Author contributions

JJ and JC designed the study. JJ, GR and KED performed the field work. JJ and ASYM collected the morphometric data. JJ conducted genetic tests. ASYM photographed the specimens and created the taxonomic drawings. JJ conducted the statistical analyses. JJ wrote the text. All authors commented on the text.

Chapter 8

Assessing the invasion potential of alien branchiobdellidans in the UK: survival, reproduction and transmission *in vivo* and *in vitro* experimental studies

CHAPTER 8: Assessing the invasion potential of alien branchiobdellidans in the UK: survival, reproduction and transmission *in vivo* and *in vitro* experimental studies

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Article in preparation for peer review

8.1 Abstract

The impact of invasive species on the recipient ecosystem can be strongly influenced by the presence of associated parasites. The majority of introduced parasites fail to establish in the new environment, but those that do are often highly virulent. Therefore, assessing the invasion potential of newly introduced parasites is a conservation priority, and understanding parasite life history traits is fundamental in this endeavour. Here, we use a series of *in vivo* and *in vitro* experiments to investigate the survival, reproduction and transmission of 2 non-native branchiobdellidan species (*Xironogiton victoriensis* and *Cambarincola* aff. *okadai*) recently discovered on invasive signal crayfish (*Pacifastacus leniusculus*) in the UK. These data were analysed using General Linear and Generalized Linear Mixed Models. *In vitro* at 15ºC, *X. victoriensis* and *C.* aff. *okadai* survived for up to 92 and 106 days respectively. Survival decreased with increasing temperature or nitrate concentration. Both species were able to tolerate a degree of dehydration, surviving up to 40 min and 3 h out of water at 15ºC for *X. victoriensis* and *C.* aff. *okadai* respectively. In terms of reproduction *in vivo*, *X. victoriensis* worms deposited one cocoon every 6.5 d, which hatched in 10-27 d (mean 18.8 d) while *C.* aff. *okadai* cocoons hatched in 10-11 d. *In vitro,* only *C.* aff. *okadai* deposited cocoons, from which hatched live juvenile worms. In terms of transmission potential, 100% of naïve signal crayfish became infested when exposed to *X. victoriensis* in the environment. These branchiobdellidans also readily transmitted interspecifically from signal to virile crayfish (*Orconectes* cf. *virilis*), indicating a generalist life-history. Overall, a high dispersal potential, fast reproduction rate and low host-specificity are likely to promote establishment of both branchiobdellidan species in the UK. Given its ability to reproduce *in vitro, C.* aff. *okadai*, is perhaps a facultative symbiont, whereas *X. victoriensis* is an obligate parasite, considering its dependency on the host to reproduce and the detrimental effect it has on host behaviour. Therefore, whilst both species

are likely to establish in the UK, *X. victoriensis* has greater potential to influence crayfish invasion success.

8.2 Introduction

Parasites are increasingly considered as key in determining the outcome of biological invasions (Prenter et al. 2004; Sargent et al. 2014), one of the main causes of biodiversity loss (Gurevitch and Padilla 2004; Didham et al. 2005). The majority of introduced parasites, however, fail to establish (MacLeod et al. 2010), and predicting those that are likely to flourish is difficult, as there is no single life-history strategy indicative of a successful invasion (Sol et al. 2012; Lockwood et al. 2013). A newly introduced parasite must be able to survive in the new environment, reproduce and disperse in order to achieve invasive species notoriety (Blackburn et al. 2011). Typically, invaders have a high dispersal ability and reproductive capacity, often involving hermaphroditism and/or asexual reproduction (MacArthur and Wilson 1967). For parasites, host dispersal, specificity and life cycle complexity are also important factors for predicting invasive status (Kennedy 1994), with those infecting a wide range of highly mobile hosts and having a simple direct life cycle being the most likely to successfully colonise.

Here we assess the invasion potential of ectosymbiotic branchiobellidans (Annelida: Clitellata). These annelids are common on crayfish throughout the Holarctic (Gelder 1999), are directly transmitted between hosts and are generalists (Govedich et al. 2009; Skelton et al. 2013) so they have a high chance of being co-introduced with non-native crayfish. Considering the large number of crayfish introductions in recent decades (Souty-Grosset et al. 2006; Holdich et al. 2014; Kouba et al. 2014; Chapter 3), the abundance and diversity of non-native branchiobdellidans in Europe are low. It is unclear whether this is due to lack of monitoring or because branchiobdellidans are in fact poor invaders.

Two species of branchiobdellidans, *Cambarincola* aff. *okadai* and *Xironogiton victoriensis*, were recently found for the first time on invasive signal crayfish (*Pacifastacus leniusculus*) in the UK (Chapter 7). Previous laboratory and field studies show that the crayfish-branchiobdellidan association can vary from mutualistic to parasitic (reviewed by Skelton et al. 2013). Consequently, these symbionts have the potential to alter the invasion success of non-native crayfish both beneficially and detrimentally. It is therefore imperative for invasive crayfish management to determine the likelihood of both branchiobdellidans persisting in a novel environment. The first step in this process is to understand the basic life history traits of these worms.

Here, we examine 5 aspects of the potential invasion ability of *C.* aff. *okadai* and *X. victoriensis*. Firstly, we assessed the persistence of worm infrapopulations on the host by monitoring worm burden over time. Secondly, as little is known about branchiobdellidan tolerance to physio-chemical conditions, we monitored their survival *in vitro* at 2 temperatures and 2 nitrate concentrations. Thirdly, we assessed tolerance to dehydration as this could impact their ability to survive overland dispersal of their hosts. Fourthly, we investigated branchiobdellidan reproduction, as little is known about cocoon deposition and development on the host (Govedich et al. 2009). Finally, we assessed 3 potential transmission routes: to a new host from the environment, among conspecifics and among heterospecifics. Overall, the current study indicates that both branchiobdellidan species have a high invasive potential given their direct life cycle, rapid transmission rates, generalist host range, tolerance to a range of environmental conditions and fairly rapid reproduction rates.

8.3 Methods

8.3.1 *Collection and maintenance of animals*

Crayfish, collected by trapping and manual searching in March - October of 2012 - 2014, were transported to the aquarium facility at Cardiff University. Signal crayfish (*Pacifastacus leniusculus*) naïve to branchiobdellidan worms were collected from 3 sites in Powys, mid-Wales: Dderw Farm Pond, Llyswen (SO138375); Rhydlydan ponds, Painscastle (SO168457); and the River Bachowey, Painscastle (SO166457). Virile crayfish (*Orconectes* cf. *virilis*) also naïve to branchiobdellidans were collected from the River Lee, London (TL370028). All recipient host crayfish were sexed, weighed (blotted dry mass), measured (carapace length) and visually inspected for signs of disease. Small crayfish (carapace length <28 mm) and any displaying signs of ill health, in the premoult stage, or missing chelae were excluded from the studies. Signal crayfish harbouring branchiobdellidans (*Xironogiton victoriensis* and *Cambarincola* aff. *okadai* identified according to Chapter 7) were collected from the River Gavenny (SO308164), South Wales.

All crayfish were maintained under a 16 h: 8 h light/dark regime in aerated, filtered 180 L tanks filled with dechlorinated water (15 ± 1^{o}C), gravel substrates and refugia. The animals were fed every 24 or 48 h on Tetra Crusta flakes and weekly 50% water changes were performed in both stock and experimental tanks. Branchiobdellidan-naïve crayfish were maintained separately from infested crayfish, and no equipment was shared between the tanks, to ensure naïve crayfish had no exposure to branchiobdellidans prior to their use in experiments. Crayfish

were given a minimum of 7 days to acclimatise to laboratory conditions before being used for experiments.

Worms were carefully dislodged from host crayfish using the edge of blunt forceps into a glass dish of distilled water. Removal from the host did not cause any visible damage or behavioural change to the worms, and they readily re-attached to the surface of the dish. Worms were examined under a dissecting microscope (x 30) with fibre optic illumination and only full-size (> 3.0 mm for *X. victoriensis*, > 8.0 mm for *C.* aff. *okadai*), apparently healthy specimens were used in experiments. *C.* aff. *okadai* is less abundant than *X. victoriensis* in the Welsh population (see Chapter 7) and more difficult to maintain in the lab, and therefore could not be tested under all experimental conditions.

To determine whether branchiobdellidans living off the host could be found in the field, benthic invertebrate samples from the River Gavenny site (n = 6) were collected in September 2012 by kick-sampling for one min using a standard net (0.25 x 0.25 m with a 0.5 mm mesh) and stored in 70% ethanol. Samples were subsequently examined under a dissecting microscope for the presence of any branchiobdellidans; however, none were detected.

8.3.2 *In vivo survival and reproduction*

To investigate the persistence of worm populations on the host, naïve signal crayfish (n = 40) were experimentally infested with *X. victoriensis* worms. Infestation intensities represented those naturally present in the field, based on crayfish size category (carapace length, mm): 28-31; 32-35; 36-39; 40-43; 44-48 infested with 21, 28, 65, 101 and 154 worms respectively (Chapter 7). Host crayfish were maintained individually in aerated 15 L plastic tanks with a plastic refuge. Crayfish were screened and the number of worms counted each week for 10 weeks. At each screening, any lost worms were replaced with new ones. If a crayfish moulted, this was recorded, and the moult was left in the tank for at least 24 h to allow worms to transfer back onto the crayfish.

To assess the time for cocoon deposition and hatching, branchiobdellidan-naïve signal crayfish were infested with a single adult worm (*X. victoriensis* n = 18, *C.* aff. *okadai* n = 20). Host crayfish were maintained individually in aerated 10 L plastic tanks with a refuge. Crayfish were inspected every 48 h for the presence of adult worms and cocoons. If a crayfish moulted, the exoskeleton was left in the tank for 24 h to allow the worm to move back onto the crayfish. Following cocoon deposition, the adult worm was removed from the host and the cocoon was examined every 48 h *in vivo* under a dissecting microscope to detect emergence of the juvenile. If the host moulted following cocoon deposition, the crayfish was removed from the tank so

that cocoon development could be monitored on the exuviae until detection was no longer possible due to disintegration of the exuviae (~ 2 weeks). The experiment was terminated when all cocoons had either hatched or there had been no change in the condition of the worm or cocoon for at least 30 days.

To assess the interval between cocoon deposition, the above procedure was repeated (using *X. victoriensis* only, $n = 16$) but adult worms were left on the host following deposition of the initial cocoon. Crayfish were examined every 48 h and the presence and location of worms and cocoons were recorded. Cocoons were examined under a dissecting microscope to determine when the juvenile worm had emerged. The experiment was terminated after 30 days.

8.3.3 *In vitro survival and reproduction*

To assess *in vitro* survival, individual worms were removed from their host and transferred to petri dishes (Dia. 50 mm) containing 10 ml water. Petri dishes were kept under 4 or 2 different conditions for *X. victoriensis* and *C.* aff. *okadai*, respectively ($n = 20$ worms per treatment). Survival of both species was investigated at 15 ± 1ºC and 20 ± 0.5ºC. Survival of *X. victoriensis* was also assessed in low (< 5 ppm) and high (100 ppm) nitrate water, but *C.* aff. *okadai* worms were only available for exposure to low nitrate water. Nitrate solutions were prepared by dissolving potassium nitrate (≥ 99.0% purity $K(NO_3)$, Sigma-Aldrich, USA) into dechlorinated tap water. Nitrate levels were tested with an API® Freshwater Master Test Kit. Worms were assessed weekly under a dissecting microscope to check for the deposition of cocoons and monitor condition until death occurred. When cocoons were detected they were transferred to a new petri dish under identical conditions and screened every 48 h using a dissecting microscope to assess hatching time and juvenile survival.

To investigate maximum survival time out of water, worms were removed from their host and subjected to varying periods of dehydration at either 15ºC (59% RH) or 23ºC (41% RH). Either 5 worms of *X. victoriensis* or *C.* aff. *okadai* were added to a petri dish containing 10 ml water ($n = 10$ petri dishes per species per temperature). Petri dishes were checked to ensure all worms were alive, active and firmly attached to the bottom of the petri dish. The water was then gently poured from the petri dishes leaving the worms *in situ*, and excess water was removed with an absorbent paper wick. Each petri dish was refilled with dechlorinated water following a set period of dehydration (1, 2, 4, 8, 15, 20, 25, 30, 40 or 50 min for *X. victoriensis*; 1, 4, 10, 20, 30, 60, 90, 120, 180 or 240 min for *C.* aff. o*kadai*; times selected following preliminary trials with each species). The worms were then left in water for 24 h at

15ºC to allow them to rehydrate and reanimate, and then the number alive in each petri dish was counted.

8.3.4 *Transmission*

To investigate indirect transmission of *X. victoriensis* from the environment to crayfish, 30 worms attached to a ceramic tile (5 cm x 5 cm) were placed in a 15 L aquarium with one uninfested signal crayfish (n = 20). The number of *X. victoriensis* worms on the crayfish was recorded after 1, 2, 4, 6, 8, 10, 12, 18 and 24 h.

To assess intraspecific host-host transmission, 'donor' signal crayfish (n = 20) were artificially infested by manually transferring 30 *X. victoriensis* worms directly onto the chelae using forceps. One donor signal crayfish was then placed in a 15 L aquarium with one sex and size matched (to within 10% carapace length, mm) uninfested 'recipient' conspecific, and the number of *X. victoriensis* worms on the recipient crayfish was recorded after 1, 2, 4, 6, 8, 10, 12, 18 and 24 h. The experiment was repeated using virile crayfish as both the donor and recipient hosts (n = 20 per treatment). To investigate interspecific transmission, the experiment was repeated using signal crayfish as the infested donor (n = 20) and heterospecific viriles as the recipients. Transmission of branchiobdellidans from virile to signal crayfish was not investigated due to insufficient animals being available. No potential host was used more than once in any experiment and all experimental crayfish were from a branchiobdellidan-naïve population.

8.3.5 *Statistical analysis*

A Generalized Linear Mixed Model (GLMM) with a gaussian error distribution and identity link was used to determine whether host size (carapace length), sex, moulting or time in the experiment had an effect on the weekly proportion of worms lost from crayfish artificially infested with natural branchiobdellidan intensities (Model 1). Interactions between sex and size and between sex and time were also included as fixed effects in the starting model. To detect any non-linear effects of time on worm loss, a spline was fitted to this variable and it was included as a random variable in Model 1. Assessment of the log-likelihood ratio was used to determine whether time was a significant random effect. Crayfish identification number was also included as a random factor in Model 1, to control for repeated measures. Following assessment of the random model, the fixed model was refined by stepwise deletions using the Wald statistic. For crayfish experimentally infested with individual worms, a Pearson's chi-

squared test was used to assess whether the number of hosts that lost their branchiobdellidans differed between *X. victoriensis* and *C.* aff. *okadai*.

In vitro branchiobdellidan survival was analysed using 2 separate General Linear Models (GLMs). Model 2a explored the effect of species, temperature and the interaction between these variables on survival, whilst Model 2b investigated the effect of temperature, nitrate and their interaction on *X. victoriensis* survival. An additional GLM was used to determine whether worm species, temperature and (log) time significantly affected the proportion of worms that survived dehydration (Model 3). Interactions between variables were not included in the model due to the limited number of replicates.

Transmission of worms to uninfested hosts was analysed using 2 separate GLMs; Model 4a investigated the effect of transmission pathway (*i.e.* environment-signal, signal-signal, virile-virile, signal-virile) and crayfish size (mean pair carapace length) on the speed of transmission (log time to first worm transfer, 10 signal-signal pairs were excluded from this analysis as they were not checked at the 1 h stage) and Model 4b the effect of transmission pathway and crayfish size on the (log x + 1) maximum number of worms transferred to each crayfish. All GLMs were minimised by stepwise deletion of insignificant terms using Analysis of Variance. For all models, visual examination of data plots and Shapiro-Wilk tests were used to check standardised residuals for normal distribution and homogeneity of variance (Thomas et al. 2013). In all tests, the level of significance was taken as $P < 0.05$. All statistical analyses were conducted in the R statistical package v2.15.1 (R Development Core Team 2012), with ASReml-R (version 3.0 package) used to conduct the Generalised Linear Mixed Model (GLMM) within the R interface.

8.4 Results

During the course of these experiments, it was evident that *Xironogiton victoriensis* tolerated laboratory conditions better than *Cambarincola* aff. *okadai*, surviving for > 5 months compared to < 2 weeks for *C.* aff. *okadai* cultures (15 ± 1°C). Although *X. victoriensis* survived better *in vivo* than *C.* aff. *okadai*, both species reproduced successfully *in vivo*. *In vitro* survival in water did not differ between the 2 species; however, *C.* aff. *okadai* survived dehydration longer. Whilst *X. victoriensis* appears to be reliant on the host to reproduce, *C.* aff. *okadai* deposited cocoons *in vitro* which successfully hatched.

8.4.1 *In vivo survival and reproduction*

Over 10 weeks, crayfish infested with *X. victoriensis* at intensities reflective of those observed under natural conditions lost, on average, 33.1% of their worm burden weekly. There was, however, a significant, non-linear relationship between time and the proportion of worms lost (GLMM, $F_{1, 334} = 121$, $P < 0.0001$), with the high initial rate of loss (mean = 56.5% in week 1) reducing over time (mean = 21.5% in week 10) (Fig. 8.1). The proportion of worms lost each week was not affected by crayfish sex, size, nor host moulting in the preceding week (all $P > 0.05$).

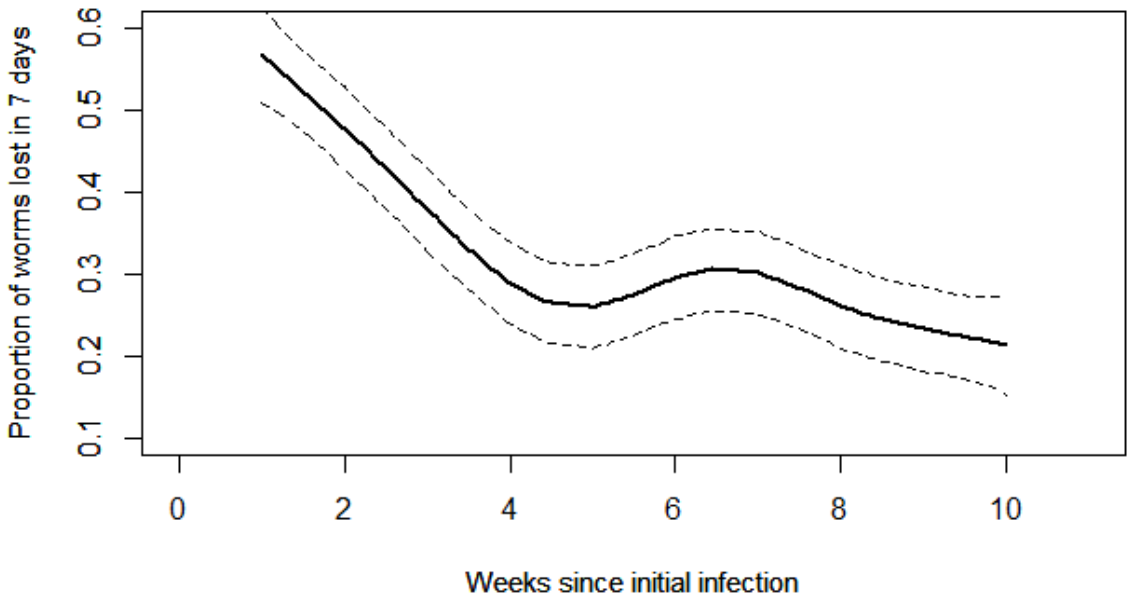

Fig 8.1. Predicted proportion of *Xironogiton victoriensis* worms that will be lost from the (initially naïve) signal crayfish host each week, as a function of time since initial infection. Dashed lines represent 95% confidence intervals.

Of the 34 crayfish ($n = 18$ where each worm was removed following cocoon deposition, $n = 16$ where worm was left in place following cocoon deposition) artificially infested with a single *X. victoriensis* worm, 32.4% lost their branchiobdellidans within the first 48 h of the experiment. Of the 23 remaining worms, 95.7% deposited cocoons on their hosts within the 30 days. The mean time to lay the first cocoon was 5 days (range 2-28) and the cocoons hatched after 10-27 days (mean 18.8) at 15 ± 1°C. Six crayfish moulted before the cocoons had hatched: these cocoons remained attached to the exuviae but none subsequently hatched. When *X. victoriensis* worms were left on the host ($n = 16$) following deposition of the first cocoon, the mean number of cocoons laid over the 30 day period was 5.7 (range 2-9), or 1 cocoon every 6.5 days, although cocoon deposition was not evenly spaced. Often, multiple cocoons were laid

over a period of a few days and this was followed by an unproductive period lasting up to 14 days before laying resumed.

Of the 20 crayfish experimentally infested with a single *C.* aff. *okadai* worm, 80% lost their branchiobdellidans within the first 48 h of the experiment. This is a significantly higher initial worm loss than for *X. victoriensis* ($\chi^2 = 5.72$, $df = 1$, $P < 0.05$). Of the 4 remaining *C.* aff. *okadai* worms, 3 laid a cocoon (on days 2, 6 and 7, respectively), while the fourth dropped off after 9 days without depositing a cocoon. Two of the 3 cocoons hatched (10 and 11 days later).

All *X. victoriensis* and all *C.* aff. *okadai* cocoons were laid on the ventral chelae or dorsal carapace, respectively, corresponding to the preferred host locations of the adult worms (Chapter 7).

8.4.2 *In vitro survival and reproduction*

Under low nitrate control conditions there was no difference in the average survival time of *X. victoriensis* and *C.* aff. *okadai* (GLM, $F_{1, 77} = 2.60$, $P = 0.11$), but both species survived significantly longer at 15ºC than 20ºC ($F_{1, 78} = 14.48$, $P < 0.0001$) (Fig. 8.2). Under the high nitrate treatment, the mean survival of *X. victoriensis* was also significantly higher at 15ºC than 20ºC ($F_{1, 77} = 111.05$, $P < 0.0001$). For *X. victoriensis*, mean survival was significantly lower under conditions of high compared to low nitrate ($F_{1, 77} = 58.11$, $P < 0.0001$). Survival times under each condition are presented in Table 8. 1.

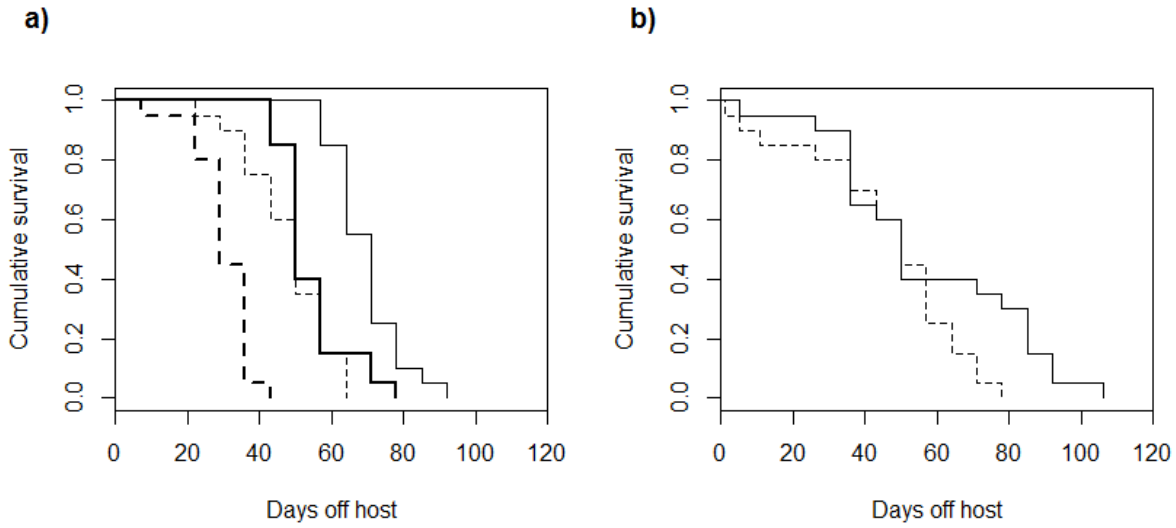

Fig. 8.2. Cumulative *in vitro* survival of branchiobdellidans at 15ºC (solid lines) or 20ºC (dashed lines) in low nitrate (light lines) or high nitrate (bold lines) water of a) *Xironogiton victoriensis* and b) *Cambarincola* aff. *okadai*.

Table 8.1. Mean (range) survival (days) of *Xironogiton victoriensis* and *Cambarincola* aff. *okadai in vitro* under various temperature and nitrate regimes (*n* = 20 per treatment).

Species	Temp. °C	Nitrate (ppm)	Mean (range) survival (d)
X. victoriensis	15	<5	69.6 (57-92)
X. victoriensis	15	100	54.2 (43-78)
X. victoriensis	20	<5	47.9 (22-94)
X. victoriensis	20	100	30.4 (7-43)
C. aff. *okadai*	15	<5	57.4 (5-106)
C. aff. *okadai*	20	<5	46.4 (1-78)

The larger *C.* aff. *okadai* worms tolerated dehydration (> 1 h at 23ºC and > 3 h at 15ºC), significantly better than *X. victoriensis* (> 15 min at 23º and > 40 min at 15ºC) (GLM, $F_{1, 37}$ = 5.73, P = 0.02), and both species had significantly better survival at 15ºC than at 23ºC when dehydrated ($F_{1, 37}$ = 11.52, P < 0.01).

X. victoriensis did not lay cocoons *in vitro*, whereas 9 out of 20 *C.* aff. *okadai* worms maintained at 15ºC and 4 out of 20 *C.* aff. *okadai* worms maintained at 20ºC deposited globular cocoons, each containing a single embryo. These cocoons were not attached to the petri dish, but some did attach to each other via a peduncle (Fig. 8.3a). Most worms (76.9%) deposited only a single cocoon, however, clusters of up to 4 cocoons were observed (Fig. 8.3b), and 1 worm laid 6 cocoons over 15 days. All cocoons were deposited within the first 23 days of the experiment. From a total of 19 cocoons, 47% (9) hatched. All living juveniles were transferred individually to a new petri dish containing dechlorinated water: 4 survived over 48 h and, of these, 2 survived 23 d *in vitro*.

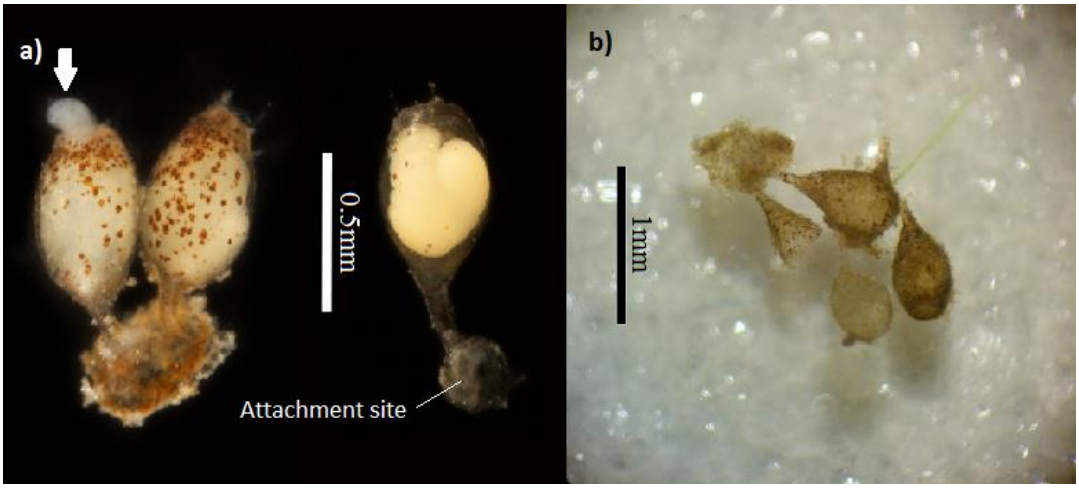

Fig. 8.3. *Cambarincola* aff. *okadai* cocoons. a) Transparent cocoons containing larvae, juvenile worm (arrow head) emerging from the far left cocoon (image courtesy of Andy Mackie, National Museum Wales), and b) Empty cocoons.

8.4.3 *Transmission*

Of the 3 transmission routes investigated for *X. victoriensis*, transmission success was greatest (100%) for environment-signal, identical for intraspecific signal-signal and virile-virile (95%), and reduced for interspecific signal-virile (70%) infestation of naïve hosts within 24 h. The time until first worm transfer was significantly dependant upon transmission route (GLM, $F_{3, 59}$ = 3.67, P = 0.02), occurring fastest between virile conspecifics and from the environment to signal crayfish. The maximum number of worms transferred also significantly varied according to transmission route ($F_{3, 76}$ = 28.59, P < 0.0001), with the highest number of worms being transferred from the environment to crayfish compared to any host-host transmission pathway. Results for each transmission route are summarised in Table 8.2. Crayfish size had no effect on either the speed of transmission or the number of worms transferred ($F_{1, 59}$ = 3.67, P = 0.06 and $F_{1, 75}$ = 0.454, P = 0.50 respectively).

Table 8.2. Proportion of naïve hosts infected, time taken for transmission to first occur and total number of *Xironogiton victoriensis* transmitted from the environment to signal crayfish, between conspecifics (signal to signal crayfish and virile to virile crayfish) and heterospecifics (signal to virile crayfish), (n = 20 crayfish pairs per treatment group).

Transmission route	Proportion (%) naïve hosts infected	Mean (range) time to first transmission (h)	Mean (range) total worms transmitted
Environment-signal	100	3.1 (1-12)	6.8 (3-12)
Signal-signal	95	6.6 (1-24)	1.6 (1-4)
Virile-virile	95	2.9 (1-18)	1.9 (1-4)
Signal-virile	70	7.2 (1-24)	2.4 (1-8)

8.5 Discussion

Here we find that 2 branchiobdellidan worms, *Xironogiton victoriensis* and *Cambarincola* aff. *okadai*, can survive for extended periods *in vitro* under different environmental conditions, including dehydration, lay 1 cocoon every 6.5 d on average *in vivo*, and readily transfer between conspecific and heterospecific crayfish pairs. For *C.* aff. *okadai in vitro* reproduction was also observed. Combined these characteristics suggest that both branchiobdellidans have a high chance of invasion success.

8.5.1 *Survival*

The population of *X. victoriensis* and *C.* aff. *okadai* in south Wales have been present for at least 3 years (Chapter 7) suggesting that both species have overcome any initial barriers associated with small founder population sizes of either worms or host crayfish. It is likely that

the persistence of both species has been aided by their ability to survive off the host for extended periods (up to 3 months at 15ºC). Both species were also able to tolerate varying temperature and nitrate conditions, although increases in temperature and nitrate reduced the survival of both species, indicating that they may be vulnerable to climate or pollution related changes in their physical or chemical environment. Little is known about the extent to which branchiobdellidans can tolerate perturbations in temperature or water chemistry. *Cambarincola* and *Xironogiton* species are reported to die within a few hours at 5ºC (Gelder unpublished data cited in DeWitt et al. 2013) and *Branchiobdella kozarovi* in Iran can survive surface temperatures up to 33ºC (DeWitt et al. 2013). At 24ºC, the branchiobdellidan *Holtodrilus truncatus* can survive up to 46 days *in vitro* (Niwa et al. 2014), shorter than the maximum survival times exhibited here (78 and 64 days for C. aff. okadai and *X. victoriensis* respectively, but at 20ºC). Mean summer surface water temperatures are currently 22.2ºC in the UK (Orr et al. 2010) but temperatures are predicted to rise by up to 0.5ºC per decade under climate change (Johnson et al. 2009). While increased temperatures may detrimentally affect branchiobdellidans it is likely that, as temperate species, their signal crayfish hosts will respond to warmer temperatures by retreating to deeper, cooler waters. The upper nitrate level used in our study (100 ppm) is twice the legal limit for UK waters, according to the 1991 Nitrates Directive (91/676/EEC), although incidences of nitrate levels rising this high have been reported (Davies 2013) and can result from pollution events. Additionally, branchiobdellidans are reportedly vulnerable to pollution from coal mines (Hobbs et al. 1967). Therefore water chemistry should also be considered as an important factor in determining branchiobdellidan persistence and dispersal in the UK.

8.5.2 *Reproduction*

The invasion potential of a co-introduced parasite is influenced by its life-cycle, host specificity and dispersal (Kennedy 1976). Branchiobdellidans are sexually reproducing hermaphrodites, which deposit fertilized cocoons directly onto the host's exoskeleton (Govedich et al. 2009). As a monoxenous species, branchiobdellidans have no requirement for an intermediate host, and this trait promotes their success as invaders (Kennedy 1994; Taraschewski 2006). However, little data exist on branchiobdellidan fecundity. The current study has demonstrated that *X. victoriensis* and *C.* aff. *okadai* tend to deposit single cocoons within a week of being transferred to a new host, and *X. victoriensis* continued to produce cocoons at a rate of approximately 1 per week. These cocoons typically hatched within 2-3 weeks at 15ºC. This is consistent with observations of *Cambarincola* cocoons (species unknown), which hatched in 10-12 days at

22ºC *in vivo* (Young 1966). This high rate of reproduction is typical of r-strategists, which are generally good at colonising new environments, and rapid population growth allows for colonisation of a new area with just 1 or 2 founding individuals (Lockwood et al. 2013). Additionally, species with a short generation time tend to exhibit faster evolutionary rates (Thomas et al. 2010), which are better able to adapt to novel environments. When isolated on the host, *X. victoriensis* continued producing cocoons despite having no opportunity to mate, indicating either self-insemination or internal sperm storage. Self-fertilising species tend to be efficient colonisers, as populations can be established by a single founding individual (e.g. Schlesinger et al. 2010) but further, longer-term experiments are required to determine the potential for self-insemination in branchiobdellidans, as well as the time until sexual maturity and the total lifetime fecundity.

Notably *C.* aff. *okadai*, but not *X. victoriensis*, deposited cocoons *in vitro* which successfully hatched. It is generally reported that branchiobdellidan cocoons will not hatch unless attached to a live host (Young et al. 1966; Sawyer 1986; Govedich et al. 2009) and, to the best of our knowledge, this is only the second report of branchiobdellidans hatching *in vitro* (Woodhead 1950, species unknown). *C.* aff. *okadai* worms were also significantly more likely than *X. victoriensis* to detach from their crayfish hosts. Clearly, *C.* aff. *okadai* is less dependent on the host than *X. victoriensis.* Govedich et al. (2009) anecdotally reports branchiobdellidans living independently in the substrate, although no detached worms were found in benthic samples collected from the branchiobdellidan-invaded site in the current study. If *C.* aff. *okadai* is not dependent on a host, this may explain the difficulty in maintaining laboratory cultures, as the detached worms are more vulnerable to predation by crayfish in the stock tanks. A number of the *C.* aff. *okadai* worms that hatched *in vitro* appeared to have been partially consumed, and some species of *Cambarincola* are known to be cannibalistic (Hobbs et al. 1967). Newly hatched branchiobdellidans have been observed to retreat into the host gill chamber (Niwa et al. 2014), and this may be an adaptive response to evade parental cannibalism.

8.5.3 *Dispersal and transmission*

In the context of parasite introductions, dispersal is largely driven by host movements (Kennedy 1976). However, this study has demonstrated that both branchiobdellidan species can survive over 3 months off the host. This creates the potential for dispersal downstream to other crayfish populations or for inadvertent transport by humans, for example on angling equipment. Worms will readily colonise new hosts from the environment, with 100% of naïve signal crayfish becoming infested when exposed to detached *X. victoriensis* worms. Detached worms are,

however, rapidly consumed by crayfish and fish (KED pers. obs.) so vulnerability to predation may hinder dispersal when detached from host. Crayfish can travel overland to scale barriers such as dams and weirs (Holdich 2003; Frings et al. 2013; Ramalho and Anastácio 2015). By demonstrating that both *C.* aff. *okadai* and *X. victoriensis* worms can survive out of water (> 3 h and > 40 min respectively) we show that the worms could tolerate these overland host excursions, allowing them to be transported between disconnected water bodies.

The spread of alien branchiobdellidans will also be promoted by low host-specificity. *X. victoriensis* worms displayed a slight preference for signal crayfish over virile crayfish, with only 70% of naïve viriles becoming infested versus 95% of naïve signal crayfish, when exposed to infested signal crayfish. Additionally, transmission was faster when worms were moving from a virile host compared to moving from a signal host. However, the maximum number of worms transmitted was not dependent upon the recipient host species. This accords with knowledge of other branchiobdellidan species, which indicates that, while some exhibit species preferences (Brown and Creed 2004), most crayfish species appear to be acceptable hosts (see Govedich et al. 2009). Indeed, some branchiobdellidan species have adopted non-crayfish hosts such as crabs and shrimps (Gelder and Messick 2006; Niwa et al. 2014). This maximises the pool of potential hosts in the environment, as there are 7 alien crayfish species as well as 1 native crayfish species in the UK (Chapter 3).

8.6 Conclusions

Risk analysis for invasive species requires information on species traits, habitat characteristics and how they match to species requirements, an estimate of exposure to the alien organism, as well as surveys of current distribution and abundance (Stohlgren and Schnase 2006). We find that both *X. victoriensis* and *C.* aff. *okadai* possess numerous traits associated with successful parasite invasion, namely, r-selection, a direct life cycle, low host-specificity and resilience to temperature and nitrate concentration fluctuations as well as dehydration. Therefore, both species have invasive potential, and thus pose a risk to the UK crayfish. In such cases where a species has been identified as an invasion threat, there is a need to characterise this risk, namely what are the potential impacts and costs of invasion to the recipient ecosystem? In the context of an invasive parasite, these effects are typically determined by the level of pathogenicity they inflict on their host, invasive or native. Therefore, understanding non-native parasite-host relationships is vital for invasive species risk assessment.

Considering its propensity to readily drop off crayfish hosts in the laboratory and ability to survive and reproduce *in vitro*, we conclude that *C.* aff. *okadai* is likely a facultative

commensal that exploits crayfish opportunistically; although further studies are need to elucidate its exact relationship with the crayfish host. Conversely, the reproductive dependency of *X. victoriensis* on the host (current study) and detrimental effect on signal crayfish competitive and foraging behaviour (Chapter 9), indicates this branchiobdellidan is parasitic. Therefore, whilst both branchiobdellidan species are likely to become established in the UK, *X. victoriensis* is perhaps more likely to have ecological consequences in terms of influencing signal crayfish invasion. This is particularly important give the widespread invasive range (Kouba et al. 2014; Chapter 3) and associated ecological problems (Chapter 2) of signal crayfish.

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Author contributions

JJ, JC, GR and KED designed the study. JJ, GR and KED performed the laboratory experiments and conducted the fieldwork. JJ and KED performed the statistical analyses. JJ wrote the text. All authors commented on the text.

Chapter 9

Reduced aggression and foraging efficiency of invasive signal crayfish (*Pacifastacus leniusculus*) infested with non-native branchiobdellidans (Annelida: Clitellata)

CHAPTER 9: Reduced aggression and foraging efficiency of invasive signal crayfish (*Pacifastacus leniusculus*) infested with non-native branchiobdellidans (Annelida: Clitellata)

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9.1 Abstract

Biological invasions are a principal threat to global biodiversity and identifying the determinants of non-native species' success is a conservation priority. Through their ability to regulate host populations, parasites are increasingly considered as important in determining the outcome of species' invasions. Here, we present novel evidence that the common crayfish ectosymbiont, *Xironogiton victoriensis* (Annelida: Clitellata) can affect the behaviour of a widespread and ecologically important invader, the signal crayfish (*Pacifastacus leniusculus*). To assess the signal crayfish–*X. victoriensis* relationship naïve crayfish were infested with an intensity of worms typically observed under natural conditions. Over a 10-week period the growth rate and survivorship of these animals was monitored and compared to those of uninfested counterparts. Complementary dyadic competition and foraging experiments were run to assess the behaviour of infested compared to uninfested animals. These data were analysed using General Linear Models and Generalized Linear Mixed Models. Whilst *X. victoriensis* did not affect the growth rate or survivorship of signal crayfish under laboratory conditions, infested animals were significantly less aggressive and poorer foragers than uninfested individuals. Through reducing aggression and foraging efficiency, infestation with *X. victoriensis* may disrupt the social structure, and potentially growth rate and/or dispersal of afflicted crayfish populations, with potential effects on their invasion dynamics. This is important given the widespread invasive range of crayfish and their functional roles as ecosystem engineers and keystone species.

9.2 Introduction

Biological invasions are a principal threat to global biodiversity (Wilcove et al. 1998), and freshwater ecosystems are particularly vulnerable to the effects of invasive species (Dudgeon et al. 2006). These threats are likely to intensify with the predicted increase in future invasion rates (Ricciardi 2001; Blackburn et al. 2011). Of all introduced species, around 1% will ultimately become invasive (Williamson et al. 1986; Williamson and Fitter 1996), and identifying the determinants of this is a conservation priority. Parasites, or the lack thereof, can alter host invasion dynamics (Prenter et al. 2004; Torchin and Mitchell 2004; Dunn et al. 2012) and in some cases are considered to be the key factor determining the outcome of species' invasions (Tompkins et al. 2003).

Most often the role of parasites in determining invasion success is considered in the context of the Enemy Release Hypothesis which postulates that escape from natural enemies facilitates the establishment and spread of non-native species (Keane and Crawley 2002). In their non-native range, introduced animals can escape over 75% of their native parasites (Torchin et al. 2004), only about 25% of which are replaced by parasites acquired from the recipient ecosystem (Torchin et al. 2003). It is unsurprising, therefore, that the role of many parasites (co-introduced or acquired from their new habitat) in controlling invaders is comparatively understudied (Mitchell et al. 2006; Sargent et al. 2014). The effects of parasites on non-native hosts may be equally as profound as those resulting from parasite absence. For instance populations of invasive rusty crayfish (*Orconectes rusticus*) in North America exist in alternate abundance states that can be at least partially explained by the presence of a trematode parasite, *Microphallus* sp., which reduces crayfish abundance and population growth (Sargent et al. 2014).

Identifying factors that allow non-natives to thrive in new environments is particularly important for crustaceans, which comprise a disproportionately large proportion of the 13 freshwater species listed among the 100 'worst' invasive species (Strayer 2010). In particular crayfish have been widely translocated for aquaculture (Strayer 2010; Holdich et al. 2014), and their invasive range now extends throughout most of Europe (Kouba et al. 2014; Chapter 3) and into Asia (Kawai et al. 2004). Invasive crayfish pose a significant threat to freshwater biodiversity and ecosystem functioning (Twadochleb et al. 2013; Chapter 2).They are host to a wide range of fungi, viruses, bacteria, protists and metazoans (Longshaw 2011), many of which may alter their invasion success if co-introduced. It is well established that the spread of North American crayfish across Europe is facilitated by transmission of *Aphanomyces astaci*, the causative agent of crayfish plague, to susceptible European crayfish, in which infection is

typically lethal (Unestam and Weiss 1970; Holdich and Reeve 1991; Kozubíková et al. 2009). In contrast, North American crayfish species are largely resistant to the disease (Unestam and Weiss 1970) and therefore gain a competitive advantage over native crayfish species.

Whilst *A. astaci* has been extensively reported, other lesser-known groups of symbionts may also affect crayfish invasion dynamics. One such group are the branchiobdellidans, ectosymbiotic annelids that have a widespread global distribution across the Nearctic and 2 disjunct regions of the Palearctic (Gelder 1999). Invasive American branchiobdellidans were first recorded in Europe on North American signal crayfish (*Pacifastacus leniusculus*) from Sweden during the 1960s (Franzén 1962) and have since been found in Austria (Subchev 2008), Finland (Kirjavainen and Westman 1999), Spain (Gelder 1999; Oscoz et al. 2010; Vedia et al. 2014), Italy (Quaglio et al. 2001; Oberkofler et al. 2002), France (Subchev 2008; Laurent 2007; Gelder et al. 2012), Hungary (Kovács and Juhász 2007), and most recently from the UK (Chapter 7). The impact of branchiobdellidans on the invasion dynamics of crayfish in these countries is however difficult to predict given the variable nature of the crayfish-branchiobdellidan relationship (Skelton et al. 2013). Although branchiobdellidans are generally considered commensals (Bishop 1968; Keller 1992; Govedich et al. 2009), their association with crayfish can vary from mutualism (Brown and Creed 2002; Lee et al. 2009; Brown et al. 2012) to parasitism (Hobbs et al. 1967; Brown et al. 2012; Rosewarne et al. 2012) depending on the host, branchiobdellidan species and density, and environmental conditions. Also, many species of branchiobdellidans have been categorized as commensals based only on crayfish growth rate and/or survivorship studies (e.g. Keller 1992), although it is known that ectosymbionts alter host behaviour in multiple ways, some of which reduce host fitness (e.g. Ravel et al. 2011). Therefore whilst branchiobdellidans clearly have the potential to influence the invasion dynamics of non-native crayfish; elucidating the nature of this effect is complex.

Here, in a series of laboratory experiments, we assessed the impact of *Xironogiton victoriensis* (Annelida: Clitellata) on the growth rate, survivorship and behaviour of their native signal crayfish hosts (Gelder and Hall 1990). Our aim was to investigate how these symbionts may influence the invasion dynamics of signal crayfish in their non-native range.

9.3 Methods

9.3.1 *Collection and husbandry of experimental animals*

In June 2013, *Xironogiton victoriensis* infested signal crayfish were collected from the River Gavenny (Abergavenny, Wales) and uninfested crayfish from the Bachowey River (Powys, Wales). All crayfish were harvested using standardised manual searches (stone turning and kick

sampling). Following capture, animals from each population were transported to Cardiff University and housed in separate 100 L tanks filled with dechlorinated water (15 ± 1°C) under a 16h: 8h light/dark regime, at a density of ca. 15 individuals/m^2. All experiments were conducted under these environmental conditions, and using only crayfish from the uninfested population. Stock tanks were supplied with gravel substrate (2 cm) and sufficient refuges (plastic tubes and plant pots) for all animals. Crayfish were fed daily with Tetra Crusta crayfish food pellets and 50% water changes were performed weekly. Crayfish with regenerating or missing chela or displaying signs of disease were not used in any experiment. Upon termination of experiments, all animals were humanely destroyed by freezing at -20°C, in accordance with the Wildlife and Countryside Act 1981.

For use in foraging efficiency trials, *Gammarus pulex* were collected from the same location as the uninfested crayfish, Bachowey River (Powys, Wales), in May 2014 using a fine-mesh dip net. These gammarids were maintained in a 60 L tank filled with dechlorinated water and housed under the same temperature and lighting conditions as the experimental crayfish. Gammarids were fed daily with a mixture of *Spirulina*, yeast and dechlorinated water.

9.3.2 *Experimental infestations with branchiobdellidans*

Worms carefully dislodged from naturally infested signal crayfish using the edge of blunt forceps into a Petri dish, were checked that they remained active and undamaged using a dissecting microscope with fibre optic illumination. Worms in good condition were then transferred on to the carapace of recipient animals using forceps, and observed to ensure that they had fully attached to the recipient crayfish. Experimental infestation loads were based on those in a naturally infested wild population of signal crayfish, which varied according to host size (Chapter 7 and see below).

9.3.3 *The effect of branchiobdellidan infestation on signal crayfish growth and behaviour*

To investigate the effects of long term exposure to branchiobdellidans on crayfish growth, individually maintained animals were weighed weekly over a 10 week period (n = 40 per treatment, sex and size matched to within 10% carapace length, CL), and then interactions between infested vs uninfested individuals were assessed over 1 day. Crayfish were housed in 15 L tanks containing a single plant pot refuge and fed every 48 h with 2 g of commercial fish food flakes. Crayfish in the infested treatment were grouped in the following size categories (CL, mm): 28-31; 32-35; 36-39; 40-43; 44-48, and infected with 21, 28, 65, 101 and 154 worms respectively, these reflect natural burdens of *X. victoriensis* on signal crayfish (Chapter 7).

Weekly, infested crayfish were screened and, if their branchiobdellidan burden declined at any point, new worms were added to maintain a constant infection intensity on each crayfish throughout the experiment. Branchiobdellidan declines were expected as crayfish commonly regulate worm densities through grooming (Farrell et al. 2014), a behaviour frequently observed during our experiments. If a crayfish moulted, the moult was left in the tank for at least 24 h to allow worms to transfer back onto the crayfish.

At the end of the 10 week experiment, dyadic competition trials were conducted between infested and uninfested crayfish in an experimental tank (L60 cm x W30 cm x D30 cm) separated into 3 compartments using a mobilised plastic divider. Prior to trials commencing, an infested crayfish, and a sex and size matched (within 10% carapace length) uninfested crayfish, were placed on either side of the divider. After 5 min acclimatisation the mobile dividers were lifted and interactions between the infested and uninfested crayfish recorded for 1 h using Micropix USB webcam cameras. The number of intraspecific interactions made by each crayfish during the trial period was subsequently recorded. It was not possible to distinguish which crayfish were infested in these webcam recordings, thus all observations were made "blind" using an identifying nail polish mark applied to the dorsal carapace to recognise individuals. The 4 types of intraspecific behaviours recorded were characterised as: i) fight - whereby a physical interaction is initiated (chelae strike/locking), ii) threat - where 1 crayfish approaches another in a threatening posture (e.g. chelae raised) but no physical contact is made, iii) retreat - where a crayfish retreats from a physical interaction (i.e. backs down from a fight) and, iv) avoid - when a crayfish moves away from an approaching crayfish but no physical interactions have taken place (i.e. the crayfish moves away from a threatening opponent). For these competition experiments, we recorded the number of worms on the infested and uninfested crayfish at the start and end of the trial respectively and the total contact duration (s) between the pair over the 1 h test period.

9.3.4 *The effect of branchiobdellidan infestation on crayfish foraging efficiency*

To determine whether short-term exposure of naïve signal crayfish to branchiobdellidans altered their foraging efficiency we experimentally infested signal crayfish (n = 25) with *X. victoriensis* and assessed their predation on gammarids, compared to uninfested controls (n = 25). Crayfish were housed individually in 10 L tanks, containing a single plant pot refuge, and allowed to acclimatise for 3 days. On Day 3 half of the crayfish were infected with branchiobdellidans and the other half sham infected by handling alone without exposure to *X. victoriensis*. Infested crayfish received 90 worms, reflecting the mean natural infection intensity

for crayfish of the size used in the experiment, 38.6-62.1 mm carapace length (Chapter 7). Following experimental infection, each crayfish was returned to its respective 10 L tank and left to acclimatise, without being fed, for 3 days prior to foraging experiments commencing. On Day 6, the refuge was removed from all tanks and 5 live gammarids (size range: 6-12 mm body length) were introduced. Latency to attack (time taken to launch the first attack, irrespective of success) and the number of gammarids each crayfish consumed was recorded at 10, 30, 60 min and 18 h. At the end of the experiment, the number of worms remaining on each crayfish was also recorded.

9.3.5 *Statistical analysis*

All analyses were conducted in the R statistical package v2.15.1 (R 2009) with Generalized Linear Mixed Models (GLMMs) being conducted using the ASReml-R (version 3.0 package within the R interface). For each model the error distribution (quasi-poisson, gaussian, poisson or Gamma) was selected by; visualizing histograms of the dependent variable, assessing residual plots as recommended by Pinheiro and Bates (2000) and, specifically for quasi-poisson models, measuring over-dispersion using the dispersion parameter, theta (Thomas et al. 2013). Non-significant terms were sequentially deleted from starting models using Analysis of Variance for General(ised) Linear Models (Crawley 2007) and the Wald statistic for GLMMs (Thomas et al. 2013), and only significant terms are reported. The fit of the refined models, was assessed using residual plots (Pinheiro and Bates 2000).

A General Linear Model with a gaussian error distribution and identity link function was used to assess whether the percentage change in weight of crayfish over the experiment was significantly different between infested (n = 36) and uninfested crayfish (n = 26 at the end of the experiment). Crayfish size (carapace length, CL mm), sex and whether or not the crayfish moulted during the experiment were included as independent variables, as well as interaction terms between infestation status and both crayfish size and moult status. A Chi square test was used to compare the number of crayfish moults in the infected (n = 36) and uninfested group (n = 26).

Generalised Linear Models with a quasi-poisson error distribution and log link function were used to assess the effect of crayfish infestation status (branchiobdellidan infested or control), sex and size (CL mm) on their behaviour. Data for each behaviour type (i.e. fight, threat, retreat, avoid) were analysed independently and models also included the total number of all behaviours performed by each crayfish as a controlling variable.

For crayfish in the infested treatment group (n = 28) quasi-poisson Generalised Linear Models (log link function) were run to assess the impact of infestation intensity (measured as the number of worms on the infested crayfish at the start of the trial) on crayfish behaviour. As infestation intensity and crayfish size (CL) were positively correlated (Pearson's correlation: t = 6.02, df = 26, $P < 0.001$) analysing them as separate independent variables could cause issues relating to collinearity (see Thomas et al. 2013). Therefore, for each behaviour, we ran 3 separate GLMs; one including crayfish size (CL) and infestation intensity as independent variables, one just including crayfish size (CL), and another just including infestation intensity. All models also included as independent variables, crayfish sex and the total number of all behaviours performed by each crayfish. All behaviour types were analysed individually. A separate Generalised Linear Model with a Gamma error distribution and a log link function was used to assess how crayfish infestation intensity (i.e. the number of worms on the infested crayfish at the start of the trial) is influenced by sex and size (CL mm).

A Kendall-Tau correlation was used to determine if the number of worms transmitted to the originally uninfested crayfish was correlated to the number of worms on the infested crayfish at the start of the trial. We also used a Kendall-Tau correlation to test whether the proportion of worms on the infested crayfish that were transmitted to the originally uninfested animal over the 1 h trial period was correlated with their total contact duration (s).

A General Linear Model with a gaussian error distribution and identity link function was used to investigate the effect of infestation status (control, n = 25, or infected, n = 25), crayfish size (carapace length) and crayfish sex on the (log transformed) latency to attack gammarid prey. A GLMM with a gaussian error structure and identity link function was used to investigate the effect of infestation status, crayfish size and crayfish sex on the number of gammarids captured over the duration of the experiment. For crayfish in the infested treatment group (n = 25), GLMMs (gaussian family, identity link) were performed to assess the effect of infestation intensity (at the end of the trial) on the number of gammarids captured. For this, 3 separate GLMMs were run because of the collinearity between crayfish size and infestation intensity (Pearson's correlation: t =2.55, df = 23, P = 0.02); one including infestation intensity, crayfish size and crayfish sex as independent variables, the second including just infestation intensity and sex, and the third including just crayfish size and sex. To control for repeated measures, both crayfish identification number and time of record (10, 30, 60 min or 18 h) were included as random effects in all GLMMs. Interactions between all independent variables were included in each initial model.

9.4 Results

9.4.1 *The effect of branchiobdellidan infestation on signal crayfish growth and behaviour*

Over the 10 week experiment, there was no significant difference in the percentage weight change (GLM, $P > 0.05$) or number of moults ($X^2 = 0.008$, $df = 1$, $P = 0.98$) between uninfested and infested signal crayfish. Smaller crayfish and those that moulted gained more weight than larger crayfish or those that did not moult (GLM, $F_{1, 63} = 97.38$, $P < 0.0001$, $F_{1, 63} = 16.78$, $P < 0.001$ respectively). There was no apparent difference in growth between male and female crayfish ($P > 0.05$).

During dyadic interactions, infested crayfish performed significantly less fight (GLM, $Deviance_{1, 53} = 313.42$, $P < 0.0001$) and threat ($Deviance_{1, 53} = 405.46$, $P = 0.02$) behaviours, and significantly more retreat ($Deviance_{1, 53} = 349.35$, $P < 0.0001$) and avoid ($Deviance_{1, 53} = 445.90$, $P < 0.01$) behaviours than uninfested crayfish (Fig. 9.1). No effects of sex or size on crayfish behaviour were detected ($P > 0.05$ for all).

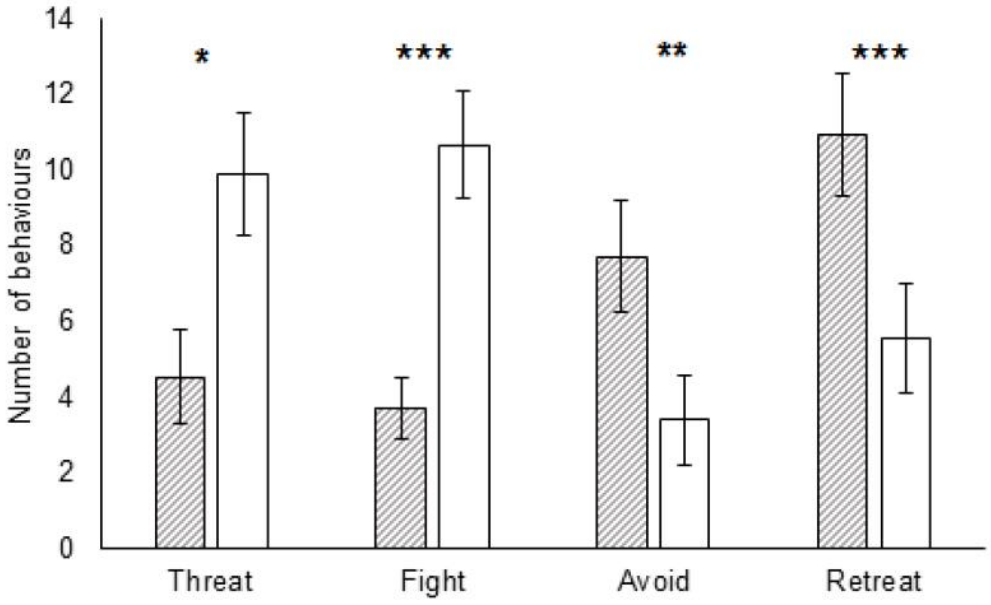

Fig. 9.1. Mean (±SE) number of threat, fight, avoid and retreat behaviours performed by *Xironogiton victoriensis* infested (hatched bars) and uninfested (white bars) signal crayfish in dyadic competition experiments. * P < 0.05, ** P < 0.01, *** P < 0.0001.

For infested crayfish the number of avoid behaviours performed by females was significantly higher than that of male crayfish ($P < 0.05$) for all 3 models (i.e. including crayfish size and infestation intensity as variables together and singularly, Table 9.1). Males performed more threat behaviours than female crayfish (*GLM*, $Deviance_{1, 25} = 200.31$, $P = 0.05$) but this effect was only significant when not controlling for infestation intensity (Table 9.1). (GLM, $LRT_{1, 25} = 6.31$, $P = 0.01$; $LRT_{1, 25} = 4.70$, $P = 0.03$, respectively). Infestation intensity was

negatively correlated with the number of crayfish avoid behaviours ($Deviance_{1, 24}$ = 116.20, P = 0.05), although this was only significant when not controlling for crayfish size (CL) (Table 9.1).

Table 9.1. The structure of Generalized Linear Models used to investigate the effects of sex, size (carapace length, CL mm) and infestation intensity on the number of avoiding, retreating, threatening and fighting behaviours performed by *Xironogiton victoriensis* (Annelida: Clitellata) infested signal crayfish (*Pacifastacus leniusculus*). For significant terms ($P \leq 0.05$) the deviance, degrees of freedom (*df*) and *P*-values are reported. In each model, the total number of all types of behaviours performed by crayfish was also included as a controlling variable, which was retained in the final refined models (after stepwise deletions of non-significant terms based on Analysis of Variance) even if not significant.

Dependent variable	Independent variables	Significant terms	Deviance	Df	P
No. of avoid behaviours	Sex, Size (CL mm), Infestation Intensity, Total Behaviours	Sex Size (CL mm) Total Behaviours	129.54 116.20 212.15	1,24 1,24 1,24	<0.01 0.03 <0.0001
	Sex, Size (CL mm), Total Behaviours	Sex Size (CL mm) Total Behaviours	129.54 116.20 212.15	1,24 1,24 1,24	<0.01 0.03 <0.0001
	Sex, Infestation Intensity, Total Behaviours	Sex Infestation Intensity Total Behaviours	119.80 116.20 195.88	1,24 1,24 1,24	0.03 0.05 <0.0001
No. of retreat behaviours	Sex, Size (CL mm), Infestation Intensity, Total Behaviours	Total Behaviours	219.89	1,26	<0.0001
	Sex, Size (CL mm), Total Behaviours	Total Behaviours	219.89	1,26	<0.0001
	Sex, Infestation Intensity, Total Behaviours	Total Behaviours	219.89	1,26	<0.0001
No. of threat behaviours	Sex, Size (CL mm), Infestation Intensity, Total Behaviours	Infestation Intensity	200.31	1,25	<0.01
	Sex, Size (CL mm), Total Behaviours	Sex	200.31	1,25	0.05
	Sex, Infestation Intensity, Total Behaviours	Infestation Intensity	200.31	1,25	<0.01
No. of fight behaviours	Sex, Size (CL mm), Infestation Intensity, Total Behaviours	Nothing	N/A	N/A	N/A
	Sex, Size (CL mm), Total Behaviours	Nothing	N/A	N/A	N/A
	Sex, Infestation Intensity, Total Behaviours	Nothing	N/A	N/A	N/A

Infestation intensity was positively correlated with the number of threat behaviours initiated by crayfish ($P < 0.01$) when infestation intensity was included in the 2 models with and without crayfish size as a variable (Table 9.1). The number of worms on infested crayfish at the start of the trial was positively correlated with crayfish size (GLM, $F_{1, 25} = 77.98$, $P < 0.001$) and higher for male than female crayfish ($F_{1, 25} = 18.30$, $P < 0.001$).

In behavioural trials at least 1 worm was successfully transmitted to 89.3% of the originally uninfested hosts within 1 h. The total number of worms transmitted to the uninfested animal was positively correlated to the number of worms on the infested individual at the start of the trial (Kendall-Tau correlation test: $z = 4.09$, $P < 0.001$) (Fig. 9.2). There was, however, no significant correlation between the proportion of worms on the infested crayfish that were transmitted to the originally uninfested animal and their total contact duration (Kendall-Tau correlation: $z = 1.36$, $P = 0.17$).

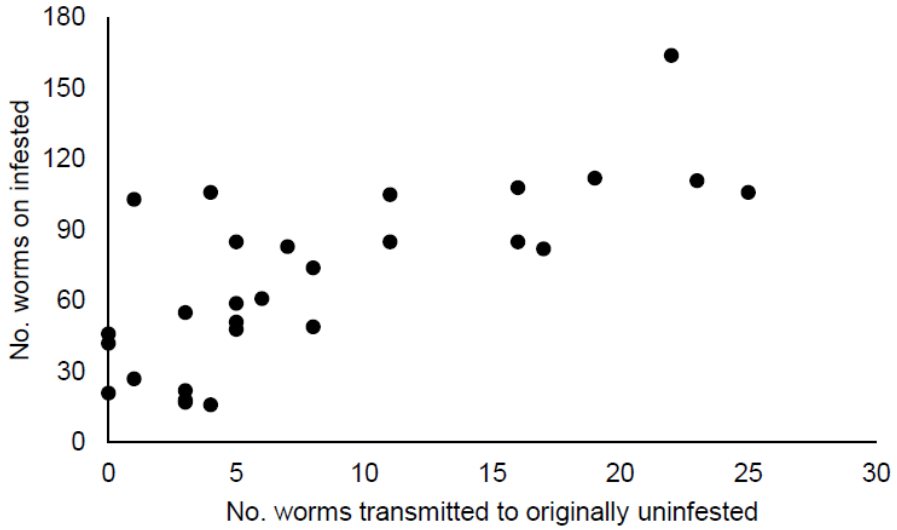

Fig. 9.2 Number of branchiobdellidans on the infested crayfish in relation to the number of worms transmitted to the originally uninfested animal during dyadic competition experiments.

9.4.2 *The effect of branchiobdellidan infestation on crayfish foraging efficiency*

Infested crayfish captured fewer gammarids than uninfested crayfish at each time point, and this difference was significant overall ($F_{1, 197} = 12.76$, $P < 0.001$; Fig. 9.3), although there was no difference in the latency to attack between these control and treatment groups ($P > 0.05$). Within the infested group ($n = 25$), crayfish with a higher infestation intensity captured fewer gammarids (Table 9.2). Infestation with *X. victoriensis* is predicted to reduce prey consumption by 19.6% for female and 22.6% for male crayfish (GLMM). Irrespective of infestation status male crayfish consumed fewer gammarids than female crayfish, with crayfish sex a significant

term in both the infestation status ($F_{1, 197} = 5.80$, $P = 0.017$) and infestation intensity (Table 9.2) models (Fig. 9.3).

Table 9.2. The structure of Generalized Linear Mixed Models used to investigate the effects of sex, size (carapace length, CL mm) and infestation intensity on the number of gammarids consumed by *Xironogiton victoriensis* (Annelida: Clitellata) infested signal crayfish (*Pacifastacus leniusculus*). For significant terms ($P \leq 0.05$) the *F*-statistic, degrees of freedom (*df*) and *P*-values are reported.

Fixed terms	Random terms	Significant fixed terms	F (incremental)	Df	P
Sex, Size (CL mm), Infestation Intensity, Size: Sex, Sex: Infestation Intensity, Size: Infestation Intensity	Time in experiment, Crayfish ID.	Sex Infestation Intensity	4.84 15.83	1,94 1,94	<0.01 0.03
Sex, Size (CL mm), Size: Sex	Time in experiment, Crayfish ID.	Sex	9.38	1,95	<0.01
Infestation Intensity, Sex, Sex: Infestation Intensity	Time in experiment, Crayfish ID.	Sex Infestation Intensity	10.30 10.36	1,94 1,94	0.03 <0.01

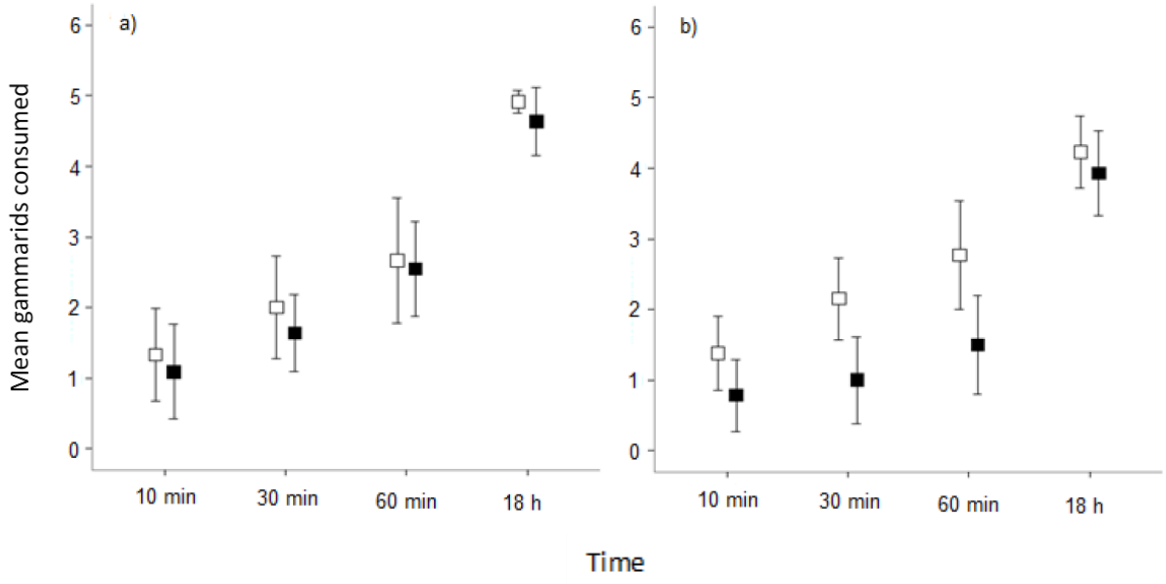

Fig. 9.3. Mean number of gammarids consumed (± 95% CI) by uninfested (unfilled boxes) and *Xironogiton victoriensis* infested (filled boxes) female (a) and male (b) signal crayfish after 10, 30, 60 min and 18 h.

9.5 Discussion

Here, we find that whilst the branchiobdellidan *Xironogiton victoriensis* did not affect the growth rate of invasive signal crayfish (*Pacifastacus leniusculus*), infested animals were less aggressive and less efficient foragers than their uninfested counterparts. These behavioural effects may reduce the overall fitness of infested crayfish, in which case *X. victoriensis* would

be considered parasitic on signal crayfish. Field studies are, however, needed to assess if the observed behavioural changes of *X. victoriensis*-infested signal crayfish translate into fitness costs in the wild. Regardless, the current study demonstrates that branchiobdellidans can alter host behaviour in multiple ways, thus determining the nature of crayfish-branchiobdellidan relationships is not straightforward.

Branchiobdellidans have variable effects on crayfish growth depending on worm species, density and environmental conditions (Keller 1992; Brown and Creed 2002; Lee et al. 2009; Brown et al. 2012). Gill frequenting branchiobdellidans, such as *Branchiobdella kobayoshi* and some *Cambarincola* species, clean epibionts from the branchial chambers promoting host respiration and growth (Brown and Creed 2002; Lee et al. 2009; Brown et al. 2012). This cleaning behaviour may, however, only be beneficial towards crayfish under conditions of high environmental fouling pressure (Lee et al. 2009). Indeed, when worm densities exceed epibiont availability, branchiobdellidans may switch to a diet of host gill tissue (Brown et al. 2012). There is some evidence that high densities of gill frequenting branchiobdellidans may reduce host growth rate (Brown et al. 2012). As *X. victoriensis* is not known to occupy crayfish gill chambers it is perhaps unsurprising that we did not detect any effects of infestation on host growth rate. A study using *Cambarincola fallax*, which primarily inhabits the subrostral region of the crayfish exoskeleton (Yoder et al. 2007), also failed to detect any effects of infestation on host growth rate (Keller 1992).

The effect of branchiobdellidans on the agonistic behaviour of their crayfish hosts has, to our knowledge, never previously been assessed. Overall, we found that branchiobdellidan infested crayfish exhibited lower aggression levels than their uninfested counterparts, which is predicted to be costly in terms of fitness, given the naturally aggressive nature of these animals (Bovberg 1956). The poorer performance of infested animals during agnostic interactions suggests that branchiobdellidan infestation may reduce the host's ability to access resources such as food, shelter and reproductive partners. The effects of branchiobdellidans on crayfish behaviour may be sex dependant with more pronounced effects noted for females. Regardless, branchiobdellidans do affect crayfish aggressiveness, which may alter development of dominance hierarchies, with potential consequences for host population dynamics.

As branchiobdellidans have a direct life cycle and are transmitted during host-host contact (Young 1966), reduced host aggression may result in decreased worm transmission rates. We, however found no evidence that the proportion of branchiobdellidans transmitted was correlated with the duration of contact between the infested and uninfested crayfish. Conversely, there was a significant positive correlation between infestation intensity (i.e. the

number of worms on the infested crayfish at the start of the trial) and the proportion of worms transmitted to the originally uninfested host. Branchiobdellidan intensity may therefore be a better predictor of transmission rate than duration of host-host contact, although presumably both factors are crucial for worm transmission in wild populations.

In terms of foraging efficiency infested animals captured, on average, fewer prey items than their uninfested counterparts. Among infested crayfish, infestation intensity was negatively correlated with the number of prey caught. By decreasing foraging efficiency branchiobdellidans may reduce long term growth in the wild where prey is limited and more spatially distributed. This may carry high fitness costs for crayfish where size is correlated to dominance (Bovberg 1956) and reproductive success (Corey 1987; Aquiloni and Gherardi 2007). Further studies are needed to elucidate whether *X. victoriensis* infestation is detrimental to crayfish fitness under natural conditions. Such studies are vital if we are to predict the effects of *X. victoriensis* infestation on signal crayfish invasion.

9.6 Conclusions

This is first report of branchiobdellidans affecting host behaviour, in this case competitive interactions and foraging of crayfish. The mechanism driving these behavioural changes is unclear, but we hypothesize that it may be driven by branchiobdellidans stimulating mechan-receptors on the crayfish exoskeleton, and thus causing interference with other behaviours (e.g. foraging and intraspecific interactions). A similar mechanism was recently proposed as being the cause of reduced foraging aptitude and predator detection in flea infested gerbilline rodents (Ravel et al. 2011). Regardless of the causal mechanism, these behavioural changes are likely to disrupt the social structure, and potentially growth rate and/or dispersal of branchiobdellidan infested signal crayfish populations in the wild. As crayfish are keystone species that interact with organisms on multiple trophic levels and alter nutrient cycling processes (Chapter 2) such changes to signal crayfish population dynamics, as well as to individual animal behaviour, may have important ecosystem level consequences. This is particularly salient considering the widespread invasive range of these crayfish (Kawai et al. 2004; Holdich et al. 2014; Kouba et al. 2014; Chapter 3).

9.7 References

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Author contributions

All authors designed the study. JJ and KED performed the laboratory experiments and conducted the fieldwork. JJ and KED performed the statistical analyses. All authors wrote and commented on the text.

Chapter 10

General Discussion

GENERAL DISCUSSION

Despite biological invasions being a principal threat to global biodiversity (Gurevitch and Padilla 2004; Didham et al. 2005), control and management resources are limited. Therefore, control efforts have been prioritized towards invasive species that have the greatest ecological impacts and/or those that affect the most speciose ecosystems. However, by the time ecological impacts of non-native species are detected these species are typically widespread and very challenging and costly to control. Therefore, preventing the dissemination of non-native species and controlling newly introduced species should be a global conservation priority (Puth and Post 2005).

The current study focussed on arguably the most ecologically damaging group of freshwater invasive species – the crayfish (Strayer 2010). Of the 644 described species, 7 are recognized non-native invasive species (Chapter 1) but worldwide research effort has been largely based on just 2 taxa: the red swamp (*Procambarus clarkii*) and signal crayfish (*Pacifastacus leniusculus*). Whilst, the majority of experimental work in this thesis focuses on signal crayfish, it does also include one of the first quantitative comparisons of the ecological impacts of different crayfish species in their native and invasive ranges (Chapter 2). The fact that invasive crayfish had a significant effect on every aquatic organism and ecosystem process investigated (Chapter 2) contributes to our understanding of the ecological problems associated with invasive crayfish worldwide. These findings enforce the need to strictly regulate the movement of non-native crayfish and control their sale in the aquarium trade. It should, however, be noted that native crayfish also had a significant effect on other invertebrates, fish, amphibians and decomposition rates (Chapter 2). Also, the impacts of invasive compared to native crayfish were only significantly greater for primary productivity and decomposition rates. These findings are particularly poignant considering that reintroductions and managed translocations of native European crayfish are already being conducted in response to population declines caused by invasive North American crayfish (Horton et al. 2009; Souty-Grosset and Reynolds 2009; Kozák et al. 2011).

Conceptually, managed translocations are similar to biological invasions (Shea and Chesson 2002) and as such they may have unintended detrimental consequences on the recipient ecosystem (Olden et al. 2010). Risk assessments for managed translocations are, however, sometimes ignored in favour of the potential benefits of conserving a heritage native species. Controversially, the findings of the current study suggest that, for native European crayfish, managed translocations may cause detrimental effects on the recipient ecosystem that outweigh any potential conservation benefits. For native species translocations/reintroductions

in general, lessons should be taken from the global problems caused by invasive species. For instance, the animals used to establish a "new" native species population are likely to be those in the best condition with low parasite burdens and possibly moved to areas with reduced predation pressure. Therefore, these populations, released from their natural enemies, may ultimately reach extremely high densities. This "enemy release hypothesis" is often considered as one of the mechanisms driving the success of species in their invasive ranges (Keane and Crawley 2002). Therefore, as a pre-requisite to all native species movements the potential detrimental impacts on the wider ecological community of the recipient ecosystem should be carefully considered.

Within the UK invasive North American signal crayfish (*Pacifastacus leniusculus*) are now widespread (Chapter 3). Considering their aggressive nature (Bubb et al. 2009) and high fecundity (Souty-Grosset et al. 2006) it is perhaps unsurprising that signal crayfish have displaced native white clawed crayfish (*Austropotamobius pallipes*) across most of the UK (Chapter 3). In response, resources have been heavily invested into trying to eradicate or control signal crayfish (e.g. Wright and Williams 2000; Peay et al. 2009). Indeed, complete eradication of signal crayfish has been achieved in some Scottish ponds through the use of natural pyrethrum (Peay et al. 2009), although this chemical is not suited for use in rivers/streams given its lethality to other aquatic invertebrates and fish (Johnson and Finley 1980). Whilst complete eradication is often the ultimate goal of signal crayfish management programs, the potential ecological effects of this should be more widely considered given that crayfish are keystone species and ecosystem engineers (Parkyn et al. 1997; Geiger et al. 2005; Crandall and Buhay 2008). Particularly in areas where signal crayfish have replaced native white clawed crayfish, the complete removal of crayfish may negatively alter the structure and function of the affected ecosystem. As, for all taxa in general, invasive species often replace their native counterparts eradication plans should firstly consider the potential knock on effects of species removal for the wider ecological community of the invaded ecosystem.

Whilst signal crayfish are currently the most successful and widespread invasive crayfish species in the UK, 6 other species have established viable wild populations (Chapter 3). Four of these are North American crayfish species, and 2 in particular, the red swamp crayfish (*Procambarus clarkii*) and the spiny cheek crayfish (*Orconectes limosus*) have caused large scale ecological problems in other European countries (Souty-Grosset et al. 2006; Gherardi 2010). However, as all 6 of these "other" invasive crayfish species in the UK currently only exist in geographically isolated populations they are largely disregarded in invasive species management programs. In parts of England signal crayfish are now facing competition from

virile crayfish (*Orconectes* cf. *virilis*), however as virile crayfish have not spread out of the River Lee, where they were initially introduced in 2004, they are generally not considered as a threat to the UK (Ahern et al. 2008). As such, little effort has been made to control the spread of virile crayfish in the River Lee. The current study shows that, as a consequence, virile crayfish now occupy about 20 km of the River Lee and, perhaps even more concerning, they are competitively superior to signal crayfish (Chapter 4). In addition, in areas of sympatry virile crayfish appear to have contracted a highly virulent strain of the pathogen, *Aphanomyces astaci* (the causative agent of crayfish plague) from signal crayfish (Chapter 6). Like signal crayfish, virile crayfish are largely resistant to *A. astaci* (Unestam and Weiss 1970; Söderhäll and Cerenius 1999 cited in Kozubíková et al. 2009; Cerenius et al. 2003), but facilitate the spread of the pathogen to native European crayfish species in which infection is often lethal (Unestam and Weiss 1970; Diéguez-Uribeondo et al. 1997; Bohman et al. 2006; Kozubíková et al. 2008; Oidtmann 2012). Therefore, the spread of virile crayfish is predicted to increase the threat invasive crayfish pose to native crayfish in the UK. It is recommended that, at this relatively early stage in virile crayfish invasion in the UK, control measures such as intensive trapping or the installation of barriers to prevent the dispersal of crayfish, are implemented. Overall, these findings show the danger of ignoring more recently introduced and less wide spread invasive crayfish species. For non-native species in general there is a far greater scope for control in the early stages of invasion (Puth and Post 2005), and the current study highlights the dangers of "ignoring" newly introduced invaders.

Native white clawed crayfish populations in the UK have declined to such an extent that since 2010 they have been categorized as endangered (IUCN, 2015). It is generally considered that the only way of ensuring the sustainability of white clawed crayfish in the wild in the UK is through the setting up of "ark sites" (Peay 2009; Kozák et al. 2011). Such managed translocations, however, require not only careful assessment of the potential consequences to the recipient ecosystem, but careful selection of the native crayfish population from which animals are sourced. One factor that may be important to consider in this selection is the extent to which the native species population in question is threatened from extirpation. Populations at higher risk of extirpation may be regarded as a higher priority for translocation that those under less threat. Crayfish plague is undisputedly one of the key threats to native crayfish in Europe, thus any populations in close vicinity to *A. astaci*-infected invasive crayfish are at high risk of extirpation. Whilst it was traditionally considered that all North American crayfish were carriers of *A. astaci* the current study shows that only around half of the 22 signal crayfish populations screened from England and Wales were infected with this pathogen, and within

these sites prevalence ranged from 3 to 80% (Chapter 5). Of the infected populations, the pathogen was genotyped in one, and found to be a highly virulent B strain (Chapter 5). As, in this study, only a fraction of the signal crayfish populations in the UK were tested for *A. astaci* increased screening for this pathogen is recommended for native crayfish conservation programs. Also, as strains of *A. astaci* differ in their virulence (Makkonen et al. 2012) pathogen genotyping should be conducted. Whilst chronic infections of *A. astaci* have so far never been observed in white clawed crayfish they have been found in closely related stone crayfish, *Austropotamobius torrentium* (see Kusar et al. 2013) therefore native crayfish from the few populations co-existing with signal crayfish in the UK should be screened for this pathogen.

Whilst the presence of *A. astaci* has undoubtedly facilitated the spread of signal crayfish in the UK other parasites may have a detrimental effect on their invasion dynamics. In this study 2 species of branchiobdellidan (Annelida: Clitellata) worms, *Cambarincola* aff. *okadai* and *Xironogiton victoriensis* are reported for the first time on signal crayfish in the UK (Chapter 7). Both species are found on signal crayfish in their native range (Yamaguchi 1933; Gelder and Hall 1990) and appear to have been co-introduced with them (Chapter 7). Whilst many co-introduced parasites fail to establish in their introduced range (MacLeod et al. 2010) the success of these branchiobdellidans is likely to be attributed to their direct life cycle, fast reproduction times and broad host specificity (Chapter 8). One of these species, *X. victoriensis*, had reduced host signal crayfish foraging efficiency and aggressiveness (Chapter 9). Further studies are recommended to determine whether these observed behavioural changes translate into fitness costs for signal crayfish in wild populations. There is a potential, however, particularly if host population growth rates are reduced as a consequence of infestation, for these worms to be used as bio-control agents for signal crayfish. The current study therefore shows that whilst many non-native parasites fail to establish in their non-native range those that do may have important impacts on the invasion dynamics of their hosts. Also, these impacts may manifest in a variety of forms, i.e. not just by causing gross pathology. In general there is a need for increased assessment of the effects of co-introduced parasites on their hosts and the potential consequences of this on host invasion dynamics.

Overall, the findings of this thesis reinforce the need to prevent the movement of non-native species, particularly those that have broad scale ecological impacts, such as crayfish. Whilst the import and sale of non-native species is already strictly regulated in many European countries (EU regulation 1143/2014), species introductions continue to occur. For crayfish, the main routes of introduction are the aquaculture and aquarium trades (e.g. Holdich 1993; Bohman et al. 2006; Chucholl et al. 2013). Introduction via the aquarium trade is of growing

concern as many exotic crayfish species are readily available in pet stores and over the internet (Chucholl et al. 2013). In Germany alone 120 non-native crayfish species are available to purchase through the aquarium trade (Chucholl et al. 2013). In many cases these crayfish outgrow aquaria and are dumped into local watercourses (e.g. Ahern et al. 2008; Chucholl and Pfeiffer 2010). Indeed, this is thought to be the route by which virile crayfish were first introduced into the wild in the UK (Ahern et al. 2008). Once introduced, non-native crayfish are often spread through anthropogenic activities e.g. being used as live bait by anglers. Increased public engagement is needed to inform anglers, and other river users, of the dangers associated with moving invasive crayfish, and non-native species in general.

The period immediately following initial introduction, before a species has established and spread, is critical in terms of control for all non-native species (Puth and Post 2005). Invasive species management programs, however, often ignore newly introduced non-native species in favour of those that are already widespread. The rationale for these actions is justified by the need to mitigate the ecological problems caused by the more widespread species, as is the case for the signal crayfish situation in the UK. This, however, does not account for the potential ecological impacts of newly introduced species if allowed to establish and the relative costs of removing isolated introduced species compared to widespread invaders. Meta-analyses, such as the one conducted within this thesis, can help predict those species that are likely to cause ecological problems when introduced, and these data can be used to inform non-native species control programs. In terms of trying to control widespread invasive crayfish further work should focus on investigating the potential of co-introduced parasites, such as branchiobdellidans, as bio-control agents.

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APPENDICES

APPENDIX I

A reference list of the 35 papers from which data was extracted for use in the meta-analysis on the impacts of crayfish in freshwater ecosystems (Chapter 2)

Angeler D.G., Sánchez-Carillo S., García G. and Alvarez-Cobelas M., 2001. The influence of *Procambarus clarkii* (Cambaridae, Decapoda) on water quality and sediment characteristics in a Spanish floodplain wetland. *Hydrobiologia, 464*, 89-98.

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Appendix I: Papers Included in Meta-analysis (Chapter 2)

Weinländer M. and Füreder L., 2011. Crayfish as trophic agents: effect of *Austropotamobius torrentium* on zoobenthos structure and function in small forest streams. *Knowl. Manag. Aquat. Ecosyst., 401*, 22.

APPENDIX II

Forest plots of individual effect sizes (*d*) for each ecosystem component with studies grouped depending on whether the crayfish used in them was native or invasive to the study region (Chapter 2).

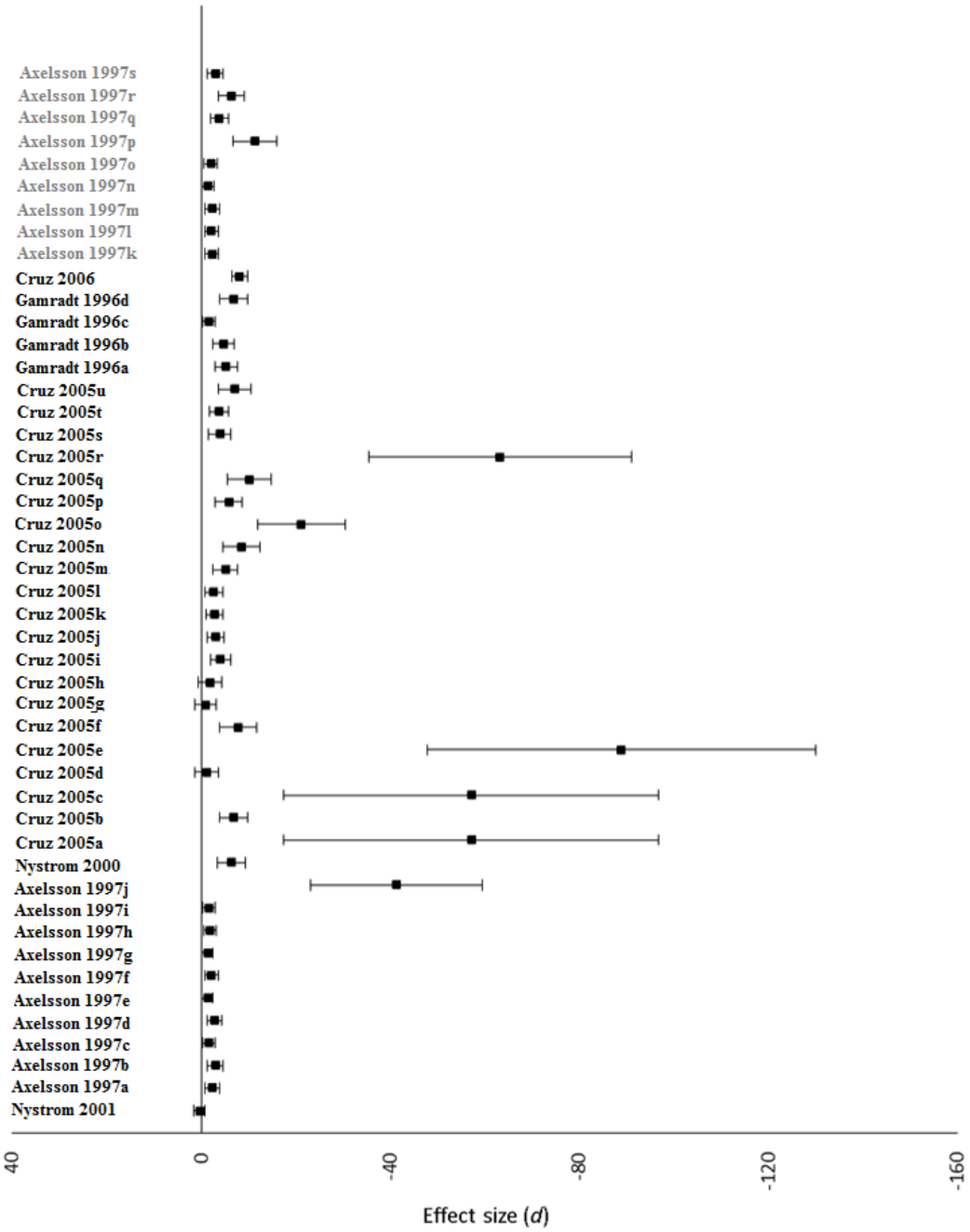

Fig. I. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on amphibian egg/larval survival rates. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

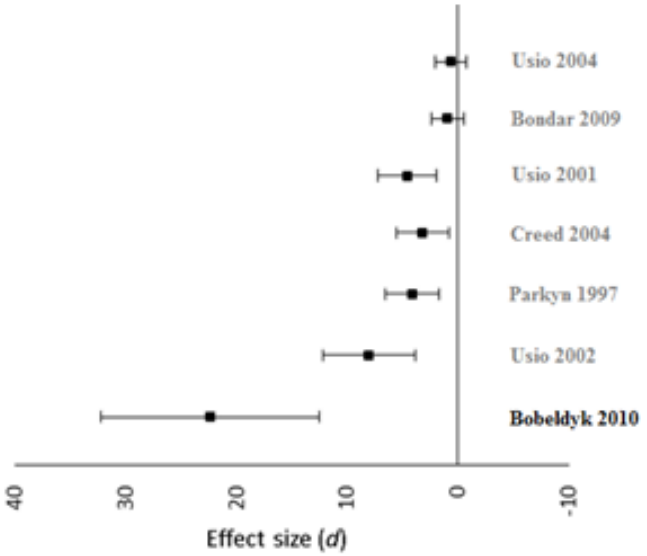

Fig. II. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on leaf decomposition rates. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

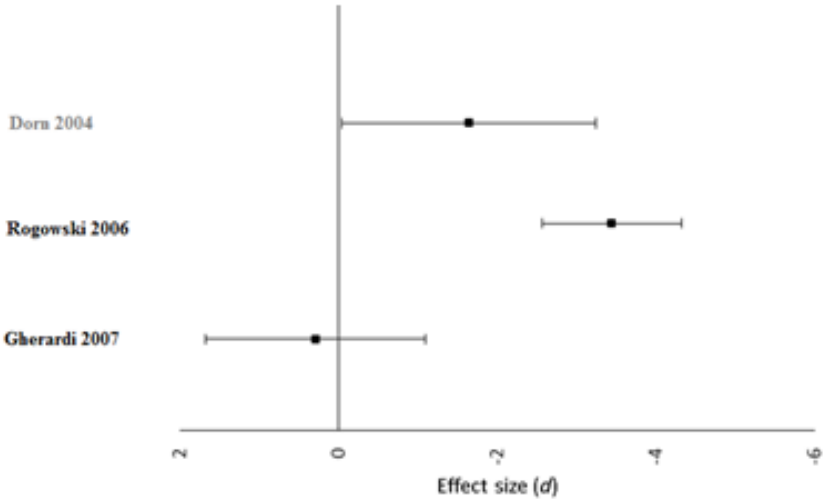

Fig. III. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on fish biomass. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

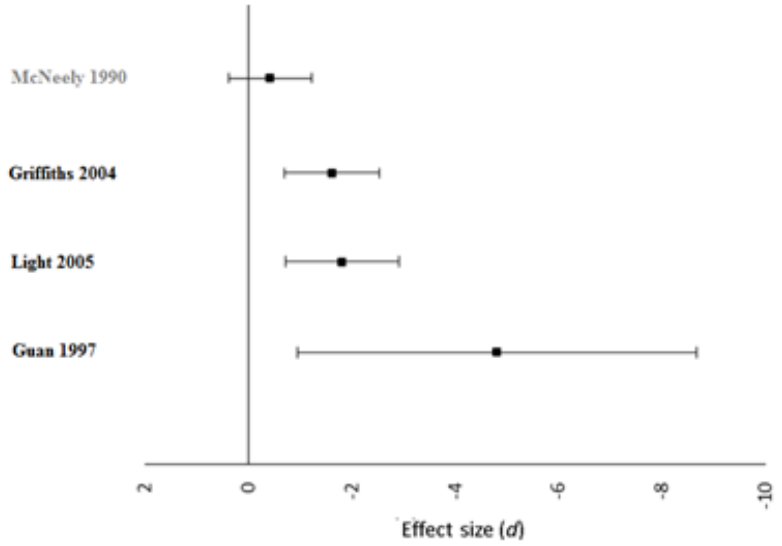

Fig. IV. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on fish refuge use. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

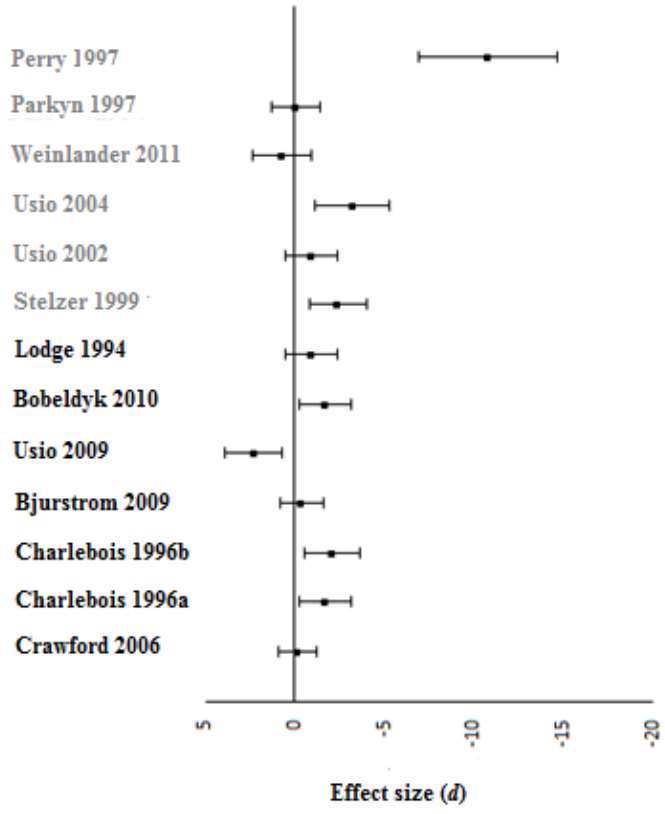

Fig. V. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on invertebrate density. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

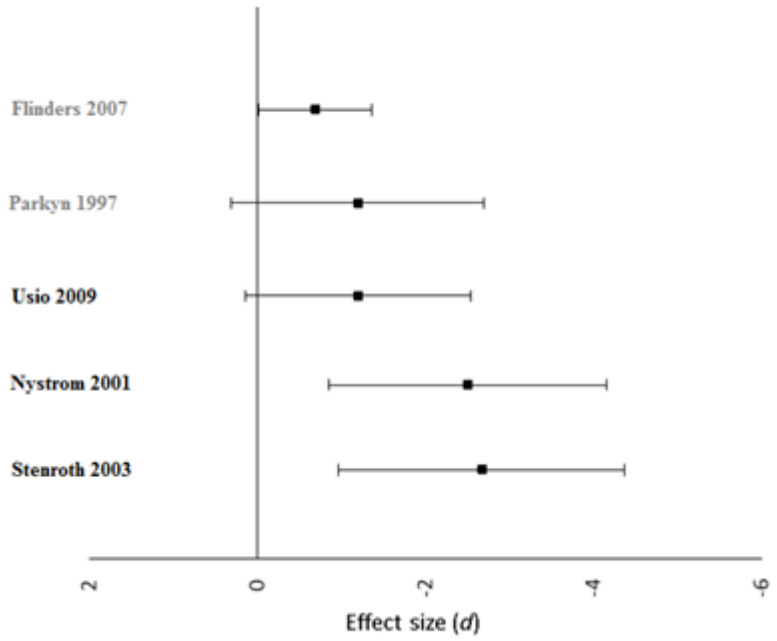

Fig. VI. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on invertebrate biomass. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

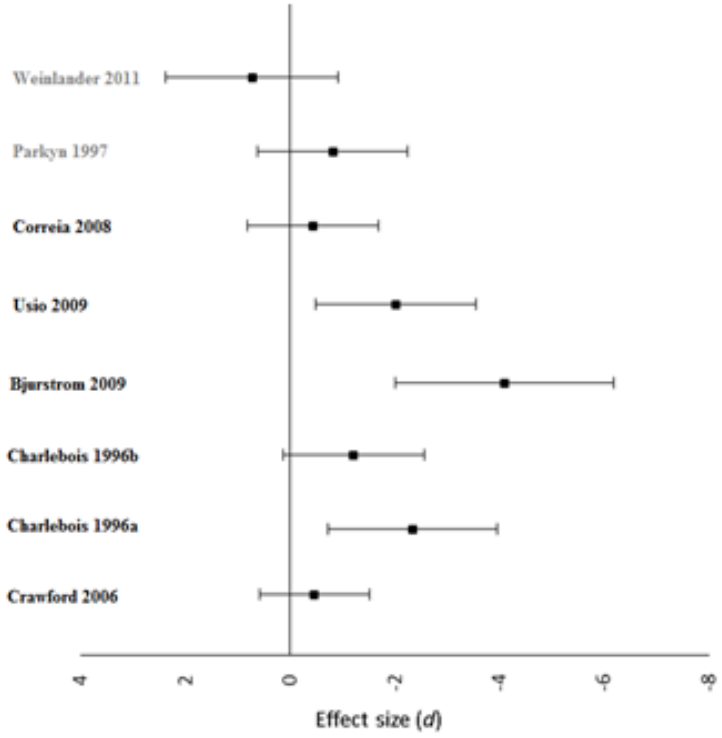

Fig. VII. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on invertebrate diversity. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

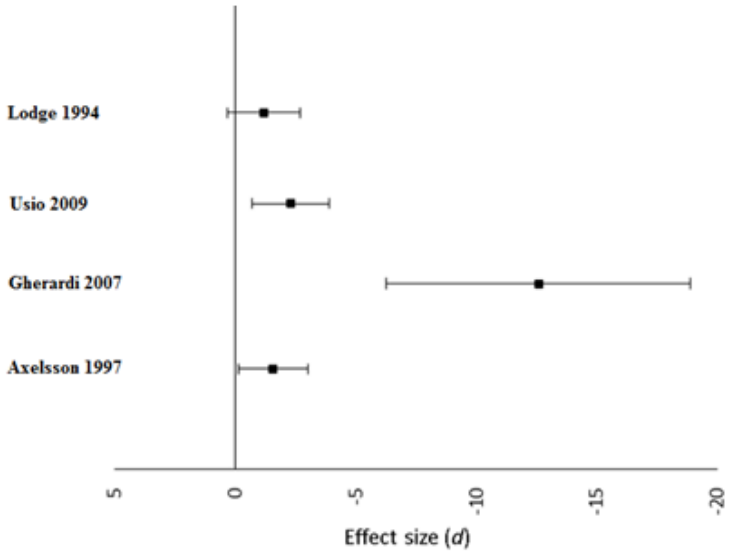

Fig. VIII. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on aquatic plant biomass. For all studies the crayfish used were invasive.

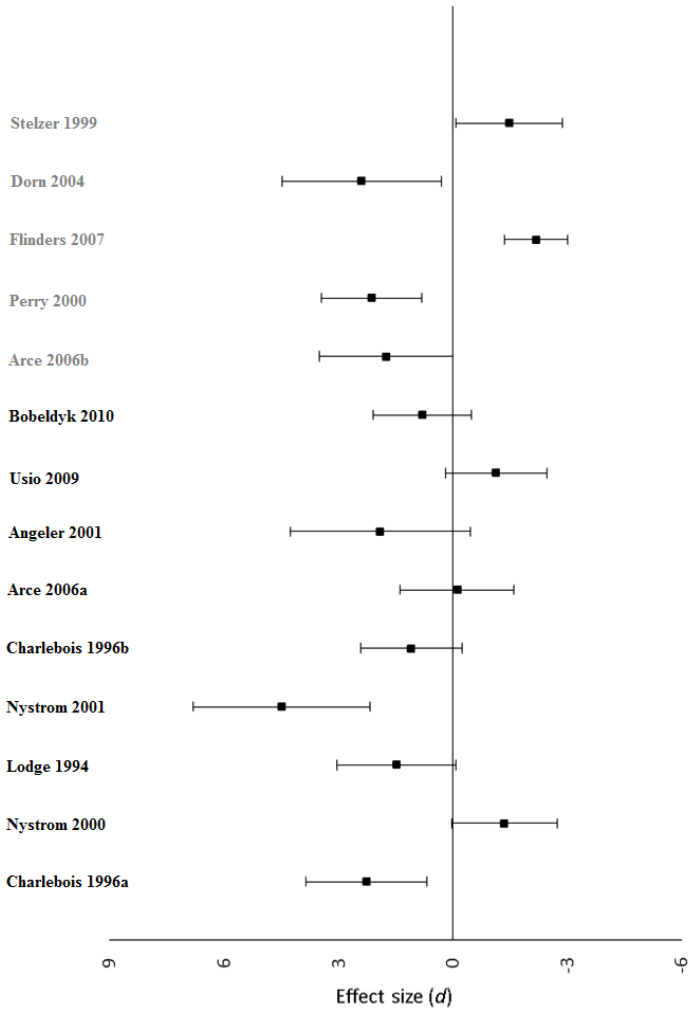

Fig. IX. Effect size, *d's* (± 95% CI) of individual studies looking at the impact of crayfish on primary productivity. Grey text indicates that the crayfish used in the study were native to the region whereas studies represented by black text used invasive crayfish.

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